DEVELOPMENT OF *IN VITRO* MODELS TO INVESTIGATE THE ANTI-INFLAMMATORY PROPERTIES OF *CYCLOPIA MACULATA* AND OTHER SOUTH AFRICAN HERBAL TEAS:

A COMPARATIVE STUDY

by

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DECLARATION

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

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ABSTRACT

Chronic inflammation is suggested to contribute to cancer development and therefore a potential target for chemoprevention. In the skin, keratinocytes and macrophages play an integral part in acute and chronic inflammation, with interleukin 1-α (IL-1α) and tumor necrosis factor α (TNF-α) as key cytokines governing this process. Green tea (*Camellia sinensis*) and the South African herbal teas, rooibos (*Aspalathus linearis*) and honeybush (Cyclopia spp.) displayed anti-inflammatory effects in mouse and human skin. To further investigate the anti-inflammatory properties of green tea and the herbal teas, rooibos and honeybush (*C. subternata and C. maculata*) herbal teas, suitable cell culture models were developed and validated utilising human keratinocytes (HaCaT) and monocyte (THP-1) derived macrophages. Aqueous extracts of the green tea and unfermented herbal teas were prepared and their chemical composition determined by high performance liquid chromatography (HPLC) and the antioxidant activity characterised utilising different antioxidant assays. Green tea and rooibos exhibited similar antioxidant activities while *C. maculata* displayed the lowest overall antioxidant activity of all the extracts, despite possessing the highest mangiferin level, the major polyphenol in honeybush. The modulation of cytokine release was studied in (i) an UVB-induced pre-exposure HaCaT model monitoring the accumulation of IL-1α and (ii) a LPS stimulated THP-1 macrophage model monitoring the TNF-α release, utilising both a pre-exposure and co-exposure extract regimens. In the pre-exposure HaCaT inflammatory model the UVB-induced IL-1α was decreased by the green tea extract while a far weaker response was obtained with the rooibos extract. Both the honeybush extracts displayed a significant effect in the reduction of IL-1α with *C. subternata* exhibiting a slight increased protection at a lower extract concentration. In the pre-exposure THP-1 derived macrophage model, green tea and the herbal tea extracts inhibited TNF-α release in a dose dependent manner in the absence of an overt loss in cell viability and apoptosis at lower extract concentrations, suggesting a typical anti-inflammatory effect. In the co-exposure model, the different extracts also exhibited an anti-inflammatory effect at the lowest concentrations in the absence of apoptosis while at higher extract concentrations the effect was masked by a decrease in cell viability and increased apoptosis. *C. maculata* exhibit differential

effects when considering the inhibition of cytokine production and, depending on the cell model, either exhibited a weaker or stronger effect when compared to *C. subternata* and rooibos. Phenolic diversity of the different teas is likely to explain the differential effects in the antioxidant assays and cell culture models with respect to the regulation of the production of the inflammatory markers. Proposed mechanism for the anti-inflammatory effects include the modulation of oxidative stress via various pathways and the subsequent down regulation of nuclear factor kappa β (NFκB) and activated protein-1 (AP-1) which are key regulators of cytokine production governing the inflammatory response.

OPSOMMING

Kroniese inflammasie van die vel kan bydra tot die ontwikkeling van kanker en is dus ’n potensiële area om te teiken in die voorkoming van velkanker. Keratinosiete en makrofage speel ’n integrale rol in akute en chroniese inflammasie van die vel en TNF-α en IL-1α is die belangrikste sitokiene wat hierdie proses inisieer. Dit is bekend dat ekstrakte van groen tee (*Camellia sinensis*) en die Suid-Afrikaanse kruietees, rooibos (*Aspalathus linearis*) en heuningbos (*Cyclopia* spp.) ‘n anti-inflammatoriese effek op die vel van muise en mense het. Om die anti-inflammatoriese aktiwitieit van groen tee, rooibos en 2 heuningbos kruietees (*C. subternata* en *C. maculata*) verder te ondersoek en te definieer is geskikte selkultuurmodelle ontwikkel en gevalideer deur gebruik te maak van menslike keratinosiete (HaCaT) en monosiet (THP-1) afgeleide makrofage. Water ekstrakte van groen tee en ongefermenteerde kruietees is voorberei en die chemiese samestelling deur hoë druk vloeistof chromatografie (HDLC) bepaal. ‘n Verskeidenheid van antioksidant bepalingstoetse is gebruik om die antioksidant aktiwiteit van die ekstrakte te meet. Groen tee en rooibos het soortgelyke antioksidant aktiwiteite getoon, terwyl *C. maculata* die swakste algehele aktiwiteit getooon het, ten spyte van die teenwoordigheid van hoër vlakke van mangiferin, die belangrikste polifenoliese verbinding in heuningbos. Modulasie van sitokiene is verder bestudeer in (i) ’n UVB-geïnduseerde vooraf-blootstelling HaCaT model, waartydens akkumulering van IL-1α gemonitor is en (ii) ‘n lipopolisakkaried (LPS)-gestimuleerde THP-1 makrofaag model, waar die vrystelling van TNF-α gemonitor is. Vir die THP-1 model is beide die voor en gelyktydige blootstelling benaderings vir die ekstrakte met LPS gebruik. In die keratinosiet model, waar die selle aan ekstrakte blootgestel is voor UVB bestraing, is IL-1α beduidend verlaag deur die groen tee ekstrak, terwyl ’n swakker reaksie gesien is met rooibos. Beide heuningbos ekstrakte het ’n beduidende invloed in die vermindering van IL-1α getoon, waar *C. subternata* ’n effense verhoogde beskerming teen selsterfte by ‘n laer ekstrakkonsentrasie toon. Blootstelling van die makrofage aan al vier ekstrakte voor LPS stimulasie (vooraf-blootstelling), het gelei tot inhibisie van TNF-α vrystelling op ’n dosis afhanklike wyse en die afwesigheid van apoptose en selsterftes by lae ekstrak konsentrasievlakke. Hierdie waarnemings dui op ’n tipiese anti-inflammatoriese effek. In die gelyktydige-blootstelling model verlaag al die ekstrakte

TNF-α vrystelling teen die laagste ekstrak konsentrasievlakke, in die afwesigheid van apoptose en met geen effek op seldood nie. Hoër ekstrak konsentrasievlakke het sitotoksisiteit en verhoogde apoptose getoon, dus was die anti-inflammatoriese effek gemaskeer. *C. maculata* toon ‘n variërende effek met betrekking tot antioksidant aktiwiteit en die bekamping van sitokien produksie, afhangend van die model wat bestudeer is. Die verskeidenheid fenoliese verbindings teenwoordig in die verskillende tee ekstrakte is waarskynlik die rede vir die effekte wat waargeneem is tydens antioksidant toetsing en selkultuurmodelle. Die anti-inflammatoriese meganismes wat deur hierdie studie voorgestel word sluit die modulasie van oksidatiewe stres via verskeie metaboliese paaie in. Modulasie van oksidatiewe stres lei tot af-regulering van kernfaktor-kappaB (NF-κB) en aktiveerderproteïen-1(AP-1), wat sleutel reguleerders van sitokien produksie tydens inflammatoriese respons is.

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CONTENTS

ABBREVIATIONS

AAPH	2,2'-azobis(2-amidinopropane) dihydro-chloride
ABTS	2,2'-azinobis 3-ethyl-benzothizoline-6-sulfonic acid
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
AP-1	Activator protein 1
BHT	Butylated hydroxytoluene
COX-2	Cyclo-oxygenase-2
DAC	*p*-Dimethylaminocinnamaldehyde
DMSO	Dimethyl sulfoxide
DPBS	Dulbeco's phosphate buffered saline
DPPH	2,2-Diphenyl-1-picrylhydrazyl
DNA	Deoxyribonucleic acid
EC	(-)-Epicatechin
ECG	(-)-Epicatechin-3-gallate
EDTA	Ethylenediaminetetraacetic acid
EGC	(-)-Epigallocatechin
EGCG	(-)-Epigallocatechin-3-gallate
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
Fe^{2+}	Iron (II)-Ferrous iron
Fe^{3+}	Iron(III)- Ferric iron
FRAP	Ferric reducing antioxidant power
GPx	Glutathione peroxidase
GSH	Glutathione
GSSG	Glutathione disulfide
GTP	Green tea polyphenols
H_2O_2	Hydrogen peroxide
HBSS	Hank's buffered salt solution
IL-1α	Interleukin-1 alpha
IL-1β	Interleukin-1 beta
IL-6	Interleukin-6
IL-8	Interleukin-8
iNOS	Inducible nitric oxide synthase
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinases
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear factor-kappa B

NO	Nitric oxide
O_2	Molecular oxygen
$O_2^{\bullet-}$	Superoxide anion
$^1O_2$	Singlet oxygen
$OH^{\bullet}$	Hydroxyl radical
ORAC	Oxygen Radical Absorbance Capacity
PGE_2	Prostaglandin E2
PMA	Phorbol 12-myristate 13-acetate
RLU	Relative light units
$ROO^{\bullet}$	Peroxyl radical
ROOR	Peroxide
ROS	Reactive oxygen species
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
TCA	Trichloroacetic acid
TNF-α	Tumor necrosis factor-α
TRAIL	TNF-related apoptosis-inducing ligand
TPA	12-O-tetradecanoylphorbol-13-acetate
UVB	Ultraviolet B

LIST OF FIGURES

Chapter 6

LIST OF TABLES

Chapter 1

GENERAL INTRODUCTION

General Introduction

Inflammation is the body's first line of defence against injury, cell damage, UVB exposure and infection (Mak and Saunders, 2006a). The skin, the body's largest organ, plays an important role as an immune organ. Keratinocytes are the principle epidermal cells and a major source of cytokines including interleukin (IL)-1, IL-6, IL-7, IL-8, IL-10, IL-12, IL-15, IL-18, and IL-20 and tumour necrosis factor-α (TNF-α) (Grone, 2002). Macrophages also play an important role in the process of inflammation and reside in all organs and tissues, usually in the locations closest to where inflammation might occur (Cavaillon, 1994). Upon stimulation they secrete a host of cytokines, including IL-1 and TNF-α that attract other immune cells to the site of inflammation (Cavaillon, 1994). TNF-α, induced by a wide range of pathogenic stimuli, induces other inflammatory mediators and proteases that orchestrate inflammatory responses (Feghali and Wright, 1997).

Acute inflammation is rapid and self-limiting, but can become chronic if it is maintained for a prolonged period (Mueller, 2006). Chronic inflammation is known to promote many diseases, including cancer (Aggarwal et al., 2006). During acute inflammation, TNF-α and IL-1α have therapeutic roles, but can mediate cancer when not regulated and secreted into circulation, as in chronic inflammation which has been linked to all steps involved in tumourigenesis and to be present in multiple tumours (Mantovani et al., 2008). Multiple intracellular signals are induced by TNF-α, including signals for cell survival through NF-κB and cell death through caspase activation (Wang et al., 1996). Thus, during chronic inflammation, the imbalance between anti-apoptotic and pro-apoptotic signalling by TNF-α may lead to the dysregulation of cell survival and proliferation, ultimately leading to tumour formation (Cooper and Caligiuri, 2003). IL-1 mediates local inflammation by promoting the expression of adhesion molecules and inducing macrophages and endothelial cells to produce leukocyte-activating cytokines, particularly IL-6, IL-8, and TNF-α (Mak and Saunders, 2006b). IL-1 and TNF-α initiate a signalling cascade that induces the gene expression and production of secondary mediators, which include cytokines and chemokines, growth factors, adhesion molecules, cyclo-oxygenase type 2 (COX-2), inducible nitric oxide synthase (iNOs) and other pro-inflammatory factors

(Borish and Steinke, 2003; Mak and Saunders, 2006b; Steinke and Borish, 2006; Williams and Kupper, 1996). This facilitates the chemo-attraction of reactive oxygen species (ROS)-producing immune cells which aid in the repair of damaged tissue (Aggarwal et al., 2006).

Plants produce polyphenols as secondary metabolites, usually for the purpose of defence against UV radiation and pathogens (Pandey and Rizvi, 2009). Polyphenols are consumed in the human diet via edible plants such as fruit, vegetables and various herbal infusions (Fraga et al., 2010; Lambert and Elias, 2010; Rice-Evans et al., 1997, 1996). Apart from a vast array of other biological effects, polyphenols have shown efficacy against chronic diseases mainly due to their ability to alter pro-inflammatory pathways via the modulation of both the production and action of inflammatory molecules (Gupta et al., 2014). Green tea (*Camellia sinensis*), known to contain high levels of catechins including epicatechin (EC), epicatechingallate (ECG), epigallocatechin (EGC) and epigallocatechin gallate (EGCG) , was shown to modulate the immune responses in skin cells (Cabrera et al., 2006). Herbal tea extracts of rooibos (*Aspalathus linearis*) and honeybush (*Cyclopia* spp.), containing a diverse group of polyphenols, have also been implicated to modulate immune responses (Joubert et al., 2008). An extract of the flavonoid-rich rooibos herbal tea modulated the immune response by stimulating the production of immunoglobulin M (IgM) associated with IL-10 production in spleen cells and red blood cells (Ichiyama et al., 2007). These effects were due to the presence of oligosaccharides and polysaccharides, rather than rooibos flavonoids. The xanthone, mangiferin, a the major polyphenol present in honeybush herbal tea, exhibits anti-inflammatory responses in reducing the production of pro-inflammatory mediators such as TNF-α and IL-6 in mouse lungs (Gong et al., 2013). The ability of the xanthones obtained from mangosteen, a Southeast Asian fruit, was shown to inhibit LPS-induced inflammation in human macrophages by reducing the expression of inflammatory genes (Bumrungpert et al., 2010). In a recent study an *in vitro* inflammatory model, utilising immortalised transformed keratinocytes, HaCaT cell line was developed (Magcwebeba et al., 2012) and showed that rooibos (*Aspalathus linearis*) and honeybush (*Cyclopia* spp.) herbal teas exhibited indirect anti-inflammatory properties by enhancing apoptosis and thereby protecting against the accumulation IL-1α following UVB exposure (Magcwebeba, 2013).

The current investigation aims to further elucidate the anti-inflammatory effects of the rooibos and honeybush teas utilising HaCaT keratinocytes and monocyte macrophage cell models. A review on acute and chronic inflammation as well as general information of different herbal teas and green tea with respect to differences in their polyphenol content are highlighted in Chapter 2. The characterisation of the chemical composition and antioxidant activity of rooibos and the different honeybush herbal tea extracts are presented in Chapter 3, highlighting their differences, using green tea as a benchmark. Chapter 4 presents the modification of a previously established *in vitro* inflammatory model, utilising immortalised transformed keratinocytes (HaCaT), by developing a pre-exposure UVB cell model to evaluate the modulating effect of the herbal tea extracts on the accumulation of IL-1α. The development of an *in vitro* macrophage inflammatory model to assess the modulating role of the different plant extracts in the inhibition of TNF-α release utilizing a pre and co-exposure approach, are presented in Chapter 5. Findings of the thesis are substantiated and contextualised with respect to the outcome of the present findings in the General Discussion (Chapter 6).

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Chapter 2

LITERATURE REVIEW

2.1. Inflammation

Inflammation is a complex series of cellular events that protects against pathogens by eliminating infectious agents, gathers intelligence for the immune system by initiating specific and long-term immunity, and repairs damaged tissue forming a crucial part of the innate immune system (Mak and Saunders, 2006a; Mueller, 2006). Acute inflammation is a rapid process, which resolves as soon as the injury or infectious agents are removed. Should resolving not occur, then the injury can lead to chronic inflammation, which is associated with a number of diseases, such as heart attacks, Alzheimer's disease and cancer (Aggarwal et al., 2006; Mueller, 2006). An acute inflammatory response is characterised by redness, swelling, heat, and pain at the site of infection or injury (Mak and Saunders, 2006a). Mediated by a complex mixture of signalling molecules, the inflammatory response is a multifactorial process that is initiated and sustained by primary cytokines, such as interleukin-1α (IL-1α) and tumour necrosis factor-α (TNF-α) (Williams and Kupper, 1996). However, the initiation of inflammation is not entirely clear. A possible trigger might be the release of proteins during cell damage, or roving immune cells that encounter bacteria in the tissues and secrete factors such as IL-1, produced by nonnuclear phagocytes, and TNF-α, produced by macrophages and T-cells (Mak and Saunders, 2006a). Both these cytokines are responsible for activating other cells of the immune response.

2.1.1. Cell Types

Hematopoietic cells are the collective name for oxygen-carrying erythrocytes and infection-fighting leukocytes, both present in mammalian blood. These cells are all derived from hematopoietic stem cells in bone marrow. Hematopoietic cells can be classified as myeloid or lymphoid. Myeloid cells include erythrocytes and leukocytes such as neutrophils, macrophages, eosinophils, basophils, and megakaryocytes. Lymphoid cells include T and B lymphocytes, natural killer cells, and natural killer T cells. Dendritic cells can arise from either myeloid or lymphoid cells (Mak and Saunders, 2006b).

Monocytes are among the largest blood cells and circulate in the blood for approximately one day before entering the tissues and serous cavities and maturing further to become macrophages. Macrophages are large, powerful phagocytes that function primarily in the tissues, engulfing and digesting not only foreign entities, but also spent host cells and cellular debris. In addition to their function in innate immunity as key phagocytes during inflammation, macrophages are also important antigen presenting cells for the T cells of the adaptive immune response. All of these functions are attributed to the ability of macrophages to produce and secrete a host of cytokines, including IL-1α and TNF-α (Cavaillon, 1994).

Neutrophils, eosinophils and basophilsare all granulocytes that harbour large intracellular granules containing microbe-destroying hydrolytic enzymes. Neutrophils respond immediately in great numbers to tissue injury. In addition to being granulocytes, they are also phagocytes. Eosinophils are leukocytes with bi-lobed nuclei and large cytoplasmic granules that stain reddish with eosin dyes. Their primary function is the removal of large parasites, with highly basic proteins and enzymes in their granules, although they can also carry out phagocytosis (Mak and Saunders, 2006b).

Basophils are circulating leukocytes with irregularly shaped nuclei and cytoplasmic granules important for inflammation, since their granules contain heparin and vasoactive amines, as well as many enzymes. These granulocytes are present in the body in very low numbers, residing primarily in the blood until they move into the tissues during an inflammatory response (Mak and Saunders, 2006a).

2.1.2. Cytokines

Cytokines are a group of soluble proteins or glycoproteins, secreted polypeptides and are produced by many cell types. They have many roles, but central to those are cell-to-cell communication and amplification as well as regulation of the inflammatory response (Peters, 1996). Cytokines can be divided into two groups, pro-inflammatory cytokines and anti-inflammatory cytokines, with the latter inhibiting the activity of the

pro-inflammatory cytokines (Dinarello, 2000). TNF-α and IL-1α are both pro-inflammatory cytokines responsible for the activation of the inflammatory response.

2.1.2.1. TNF-α

TNF-α is produced by numerous immune cells in response to an assortment of activating stimuli (Wang et al., 2003). TNF-α biosynthesis is a tightly controlled process and is regulated at many different levels to ensure low basal levels in dormant cells, though TNF-α is capable of rapid and significant up-regulation in activated cells (Beutler et al., 1985). The uncontrolled biosynthesis of TNF-α has been implicated in many human diseases of systemic inflammation, including sepsis, arthritis and Crohn's disease (Wang et al., 2003). During acute inflammation, TNF-α has a therapeutic role, but can mediate cancer if not regulated and secreted into circulation, as in chronic inflammation which has been linked to all steps involved in tumourigenesis and to be present in multiple tumours (Mantovani et al., 2008). Multiple intracellular signals are induced by TNF-α, including signals for cell survival through NF-κB and cell death through caspase activation (Wang et al., 1996). Thus, during chronic inflammation, the imbalance between anti-apoptotic and pro-apoptotic signalling by TNF-α may lead to the dysregulation of cell survival and proliferation, ultimately leading to tumour formation (Cooper and Caligiuri, 2003)

2.1.2.2. IL-1

IL-1 is the term for two polypeptide mediators (IL-1α and IL-1β) that are among the most potent and multifunctional cell activators described in immunology and cell biology. The spectrum of action of IL-1 encompasses cells of hematopoietic origin, from immature precursors to differentiated leukocytes, vessel wall elements, and cells of mesenchymal, nervous, and epithelial origin (Dinarello, 1994, 1996). Induction of secondary cytokines, including IL-6, colony-stimulating factors (CSF) and chemokines, are involved in many of the *in vitro* and *in vivo* activities of IL-1. During the acute inflammatory response, low levels of IL-1β are present, while higher levels occur during chronic inflammation which may cause tissue damage and tumour formation. IL-1α, however, have two types in existence to fulfil different functions. Membrane bound IL-1α activates immune cells responsible for the

destruction of malignant cells, while cytosolic pro-IL-1α plays a role in cell proliferation that can lead to tumour formation (Luheshi et al., 2009).

2.1.3. Inflammation in the development of cancer

The first findings of a leukocyte infiltrate in tumour tissues concluded that there is a functional connection between inflammation and cancer (Mueller, 2006).

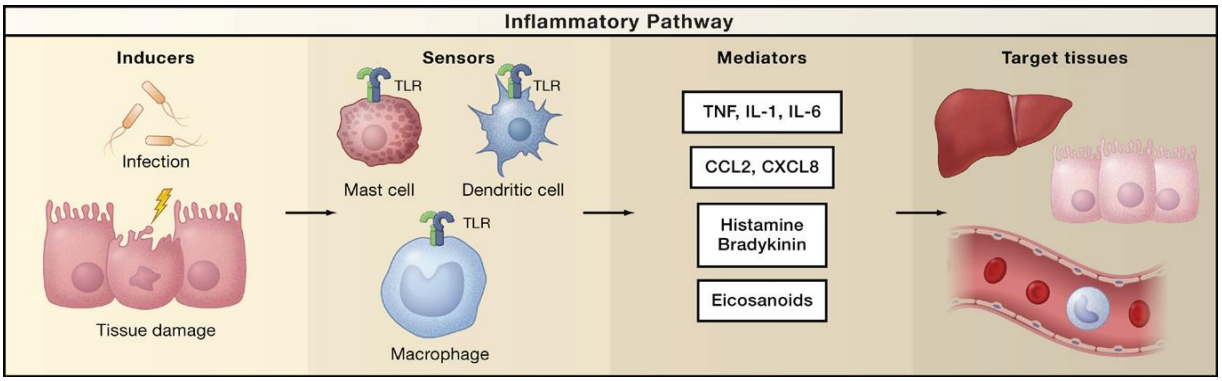

Fig. 2.1 Illustration of the inflammatory process (Medzhitov, 2010)

According to Medzhitov (2010), the typical inflammatory response consists of four components. These components are inflammatory inducers, the sensors that detect them, the inflammatory mediators induced by the sensors, and the target tissues that are affected by the inflammatory mediators (Fig. 2.1). In the case of an injury in the absence of infection, acute inflammation promotes tissue repair and helps to prevent colonization of the damaged tissues by opportunistic pathogens.

The acute inflammatory response is normally terminated once the triggering insult (an inducer) is eliminated, the infection is cleared, and damaged tissue is repaired. Termination of the inflammatory response involves the transition back to the homeostatic state and is an active, highly regulated process. This process is known as resolution of inflammation. If the trigger for the inflammation is not removed, resolution is not induced and chronic inflammation occurs (Medzhitov, 2010).

Chronic inflammation has been linked to an increased risk of cancer, by promoting tumour progression and supporting metastatic spread (Aggarwal et al., 2006; Mantovani et al., 2008; Medzhitov, 2010; Multhoff et al., 2011; De Visser et al., 2006). In the initial phase of tumour development, inflammatory mediators such as cytokines, reactive oxygen species (ROS), and reactive nitrogen species (RNS) derived from tumour-infiltrating immune cells induce epigenetic alterations in pre-malignant lesions and silence tumour suppressor genes (Multhoff et al., 2011). During tumour promotion, immune cells secrete cytokines and chemokines that act as survival and proliferation factors for malignant cells.

2.1.4. ROS production during inflammation

Molecular oxygen is the main source of ROS in cells via two different mechanisms: (1) the absorption of energy from physical irradiation, which leads to the formation of singlet oxygen ($^1O_2$) or (2) during the metabolic processes from the convertion of superoxide anion ($O_2^{-\bullet}$) to water (H_2O). The electron transport chain in the mitochondria, NADPH oxidase system from inflammatory cells, cytochrome P450, xanthine oxidase and peroxisomes (Leonarduzzi et al., 2010; Nagata, 2005) are other major sources that generate ROS. An increase in ROS occurs during inflammation from the increase in respiration in immune cells (Van Heerebeek et al., 2002). ATP is generated from ADP and inorganic phosphate to create energy for the continued function of the immune cells. This process is mediated by the electron transport chain, which creates an electrochemical proton gradient that is utilized by ATP synthase to produce ATP (Valko et al., 2007). During the electron transport chain some electrons “leak” out from complex I and complex III resulting in the incomplete reduction of oxygen yielding a superoxide anion. Myeloperoxidase release from the azurophilic granules in phagocytes are the catalysts for the formation of anti-microbial hypochlorus acid (HOCl) from hydrogen peroxide and chloride during the inflammatory process. External stimuli, such as UV radiation, phorbol 12-myristate 13-acetate (PMA) and calcium ionophore can aggravate ROS production. The antioxidant mechanism available to combat the production of ROS, subsequently, cannot “keep up” and leads to oxidative stress (Nagata, 2005). This

creates the optimal conditions for ROS to damage cellular macromolecules such as DNA, proteins and lipids.

2.2. Polyphenols

Plants produce polyphenols as secondary metabolites involved in a wide range of processes. Polyphenols are consumed in the human diet via edible plants such as fruit, vegetables and various teas, supplementing the diet with over 8000 different polyphenols (Fraga et al., 2010). These compounds consist of one or many hydroxyl groups attached to a benzene ring and can be divided into four classes based on the nature of their carbon skeletons, namely phenolic acids, flavonoids, stilbenes and lignans (Scalbert et al., 2005).

Flavonoids have a common C6–C3–C6 structure (Fig. 2.2) consisting of two aromatic rings (A and B) linked through a three carbon chain, usually organized as an oxygenated heterocycle (ring C) (Fraga et al., 2010; Harborne and Baxter, 1999; Rice-Evans et al., 1996). Due to variations in ring C, flavanoids can be divided into further subclasses (Tsao, 2010) based on their structural characteristics including the unsaturation, oxidation and variation in number and arrangement of hydroxyl groups, as well as the nature of alkylation and glycosylation (Dai and Mumper, 2010). Flavones, flavonols, flavanones and flavanonols are the most common subgroups in all plants and can be found in a variety of dietary sources.

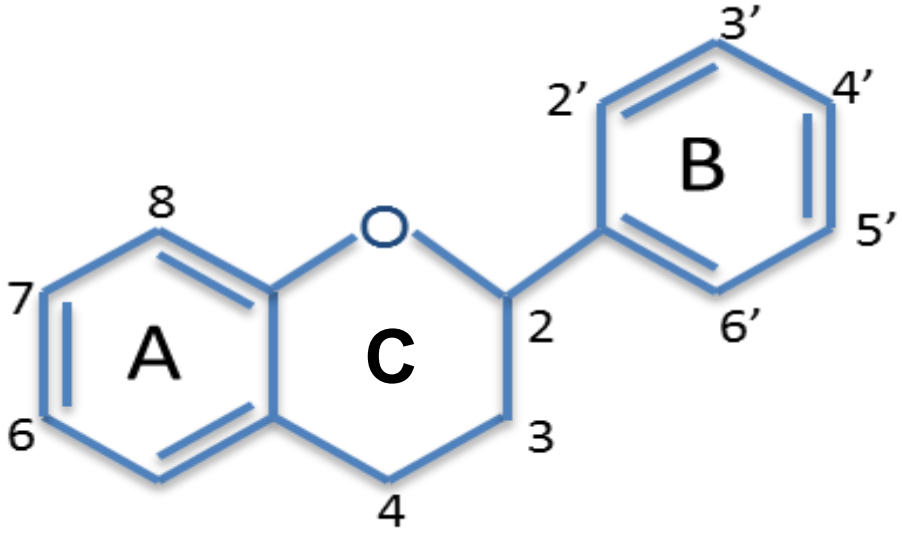

Fig. 2.2 Flavone backbone structure and numbering pattern common for flavonoids. (Harborne and Baxter, 1999)

Camellia sinensis (green tea) is rich in flavanols, of which the most prevalent are catechin, epicatechin (EC), epicatechin gallate (ECG), epigallocatechin (EGC), and epigallocatechin gallate (EGCG) (Fig. 2.3) (Cabrera et al., 2006; Cavet et al., 2011), which exhibit antioxidant effects by inhibiting ROS formation and scavenging free radicals (Tipoe et al., 2007). Green tea catechins have been established as the active compound responsible for the beneficial effects of green tea (Cabrera et al., 2006; Cavet et al., 2011; Katiyar et al., 1999a; Lambert and Elias, 2010; Suzuki et al., 2012; Tipoe et al., 2007).

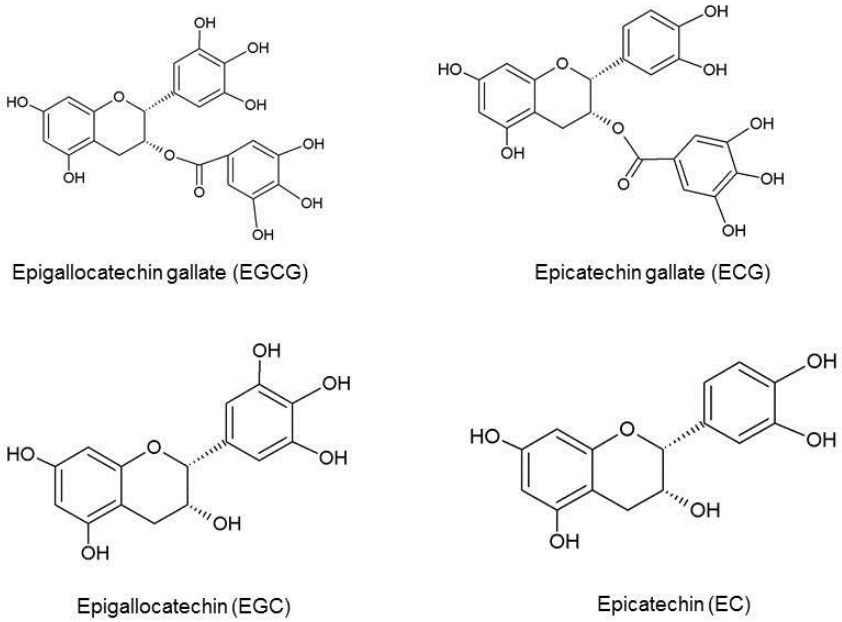

Fig. 2.3 Chemical structures of the polyphenols present in green tea.

Aspalathus linearis (rooibos) is considered to be rich in dihydrochalcones (DHC), the most prevalent of which are the C–C linked dihydrochalcone glucoside, aspalathin and the 3-dehydroxy dihydrochalcone glucoside, nothofagin (Joubert, 1996; Joubert et al., 2008) (Fig. 2.4). Both are very rare and to date aspalathin has been identified in rooibos only. Minor flavonoids consisted of the flavonols, rutin, isoquercitrin and hyperoside and the flavones, orientin and iso-orientin. Its flavonoid composition uniquely contains aspalathin, nothofagin and a rare β-hydroxydihydrochalcone.

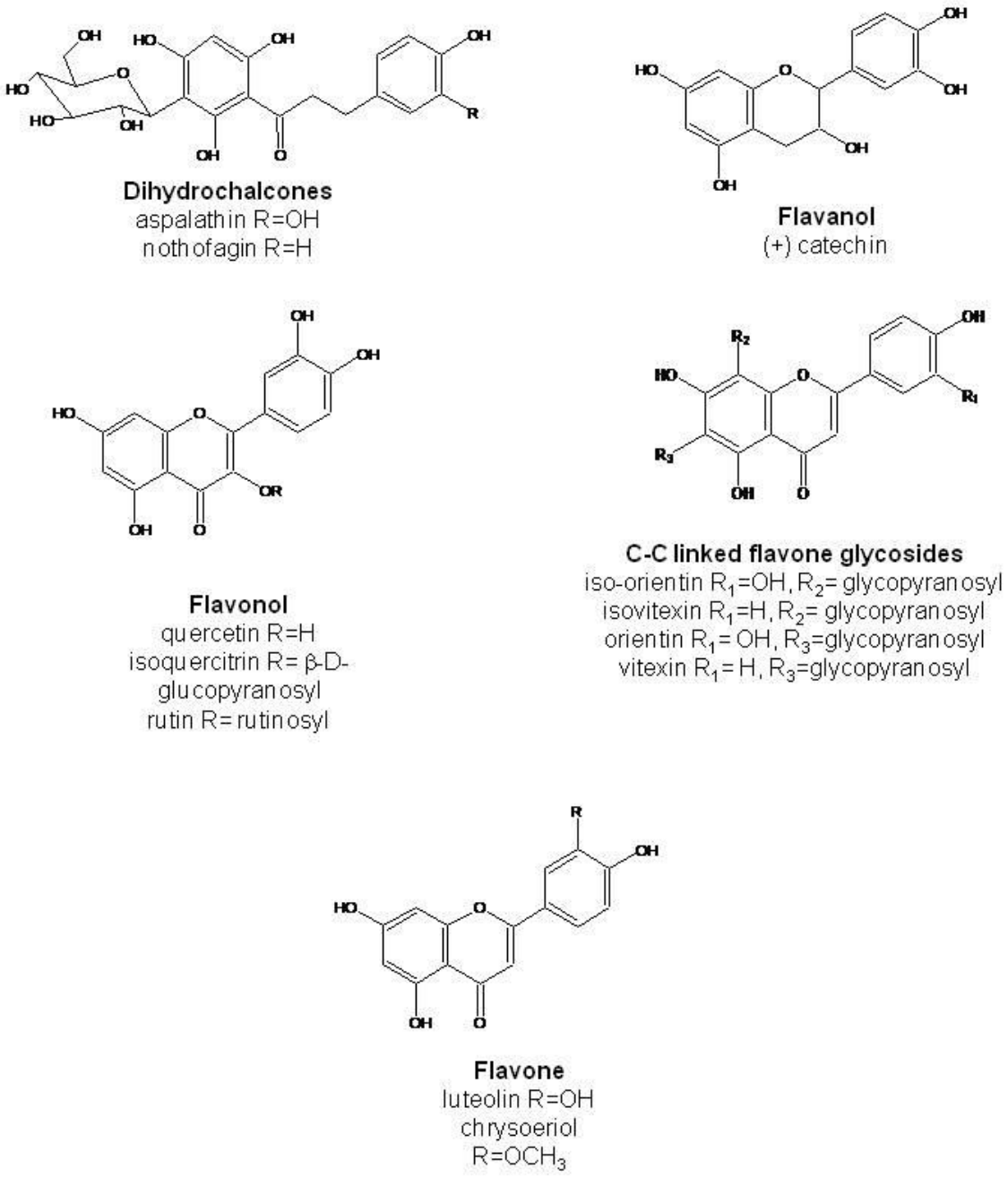

Fig. 2.4 Chemical structure of polyphenols present in rooibos.

Although there are limited reports about the honeybush species (*Cyclopia* spp.), it is known that their polyphenolic profile differs from green tea and rooibos. The

xanthones mangiferin and iso-mangiferin, and flavanones, such as hesperidin (Fig. 2.5) are the most prevelant polyphenols and present in all of the *Cyclopia* spp (De Beer et al., 2012; Joubert and de Beer, 2011). Other polyphenols include the flavones, luteolin and scolymoside with minor constituents including the benzophenones and dihydrochalcones.

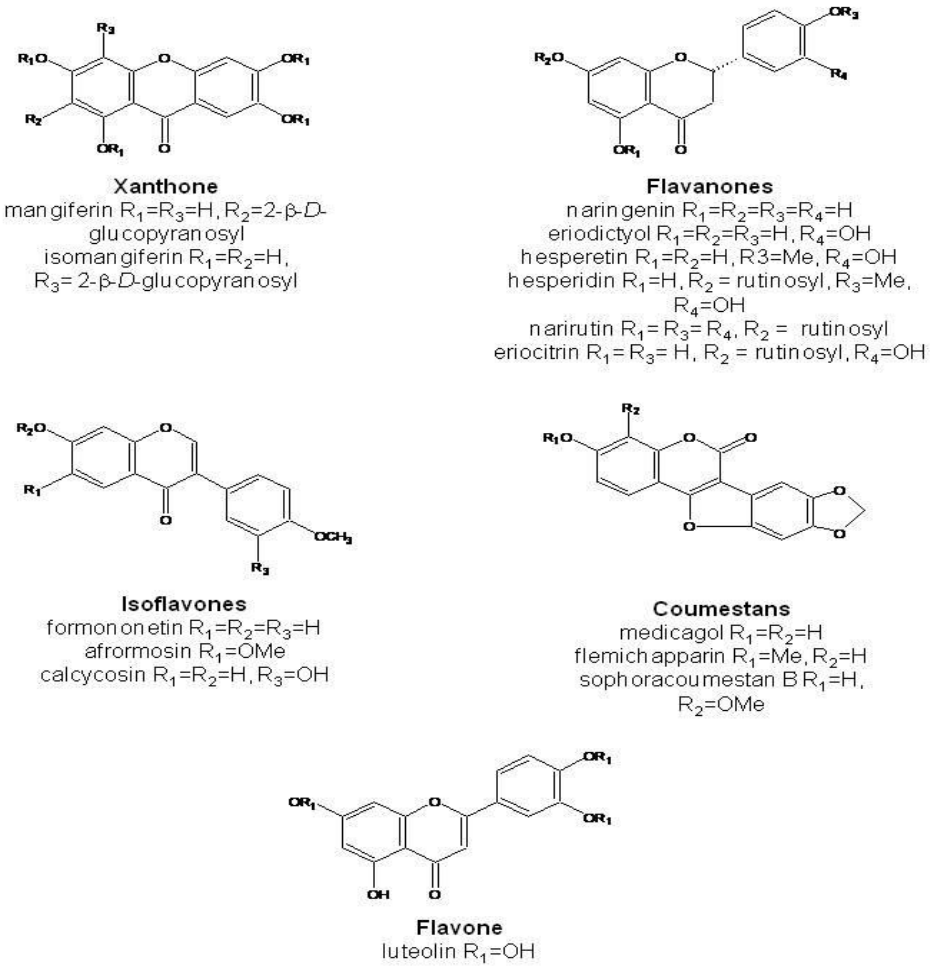

Fig. 2.5 Chemical structure of polyphenols present in honeybush.

2.2.1. Biological properties

2.2.1.1. Antioxidant properties

The antioxidant ability of polyphenols allows these molecules to protect cells from oxidative stress resulting from hydrogen or electron donation reactions, and modulate enzymes related to oxidative stress, such as superoxidase dismutase, glutathione peroxidase and glutathione reductase (Lambert and Elias, 2010; Vayalil et al., 2003). The ability of the polyphenols to delay or inhibit the oxidation of macromolecules at lower concentrations than the substrate being oxidized determines their efficiency as antioxidants, which also depends on their chemical structures (Apak et al., 2007; Rice-Evans et al., 1997, 1996).

2.2.1.2. Pro-oxidant properties

The selective toxicity of polyphenols towards tumour cells, increases their pro-oxidative state and causes oxidative damage, which leave the cells more vulnerable to apoptosis induced by ROS (Korkina et al., 2008). Lee et al (2010) have shown the ability of quercetin to generate sufficient ROS in cancer cells for the induction of apoptosis. It is also thought possible that the pro-oxidant activity of polyphenols could exhibit a beneficial function biologically (Procházková et al., 2011). Arakawa et al. (2004), for example, explains that EGCG has bactericidal activity and promotes apoptosis which can be attributed to the ability of the polyphenols to reduce O_2 to yield H_2O_2.

2.2.1.3. Anti-inflammatory properties

Green and black tea extracts and polyphenols have been shown to have anti-inflammatory properties by numerous *in vitro* and *in vivo* studies (Chatterjee et al., 2012; Das et al., 2002; Katiyar, 2003; Katiyar et al., 1999a; Tipoe et al., 2007). Consumption of green tea polyphenols have protective effects against inflammation in skin carcinogenesis by inhibiting the gene expression and production of various pro-inflammatory cytokines (IL-1α, IL-1β, TNF-α and IL-12) and other inflammatory

mediators (Katiyar, 2003; Katiyar et al., 1999a, 1999b). Green tea, as well as a host of its polyphenolic constituents (EGCG, EGC, ECG and EC) have been shown to modulate the immune responses (Cabrera et al., 2006). These compounds were effective against chronic diseases due to their ability to modulate the production and action of inflammatory molecules (Gupta et al., 2014).

The anti-inflammatory effect of rooibos extracts is yet to be established, though there are numerous studies supporting the ability of the polyphenolic constituents of rooibos to inhibit inflammation. Orientin-2”-O-galactopyranoside, structurally similar to orientin, has been shown to inhibit the NF-κB and extracellular signal-regulated kinase (ERK) pathways and subsequently reducing the production and/or release of pro-inflammatory cytokines in mouse brain cells (Zhou et al., 2014). The rooibos flavonol, quercetin has been shown to inhibit the activation of NF-κB (Hamalainen et al., 2007). Luteolin, a minor flavone of rooibos and honeybush, inhibits inflammation by inhibiting both thromboxane and leukotriene synthesis (Odontuya et al., 2005).

Mangiferin, which is the major polyphenol present in honeybush herbal tea (*Cyclopia* species), exhibits inhibitory effects on inflammatory responses, in reducing the production of pro-inflammatory mediators such as TNF-α and IL-6 (Gong et al., 2013). The ability of xanthones from mangosteen, a Southeast Asian fruit was shown to inhibit LPS-induced inflammation in human macrophages by reducing the expression of inflammatory genes (Bumrungpert et al., 2010). The flavanone hesperidin exhibited anti-inflammatory activity in mice by reducing of the release of pro-inflammatory cytokines such as TNF-α, IL-6, IL-4 and IL-10 in mice (Tamilselvam et al., 2013).

2.3. The role of tea/herbal teas against disease development

Tea has been consumed in China to promote health and longevity since 3000 BC. In South Africa only two Cape fynbos plants, rooibos and various species of honeybush, have been successfully commercialised.

2.3.1. Green tea

Green tea is one of the most studied and promising group of natural compounds against skin cancer are the catechins from green tea (Katiyar et al., 1999a). The beneficial health effects of these tea polyphenols have been demonstrated in various cell culture systems and human studies, indicating the potential use of these compounds in strategies for the prevention of cancer development (Cavet et al., 2011; Katiyar, 2003; Katiyar et al., 1997, 1999a, 1999b; Tipoe et al., 2007). Green tea polyphenols have cytotoxic effects resulting from their pro-oxidant activity which can lead to cell cycle arrest and apoptosis in cancer cells (Korkina et al., 2008; Lambert and Elias, 2010). Tipoe et al. (2007) showed that EGCG reduces the expression and production of the inflammatory mediator, COX-2 and the nitrogen radical producing inducible nitric oxide synthetase (iNOS).

2.3.2. Rooibos

Rooibos is a member of the legume family of plants. It is only grown in a small area in the Cederberg region of the Western Cape Province. Rooibos was initially used by the Khoi-San for medicinal use before it was rediscovered by Dutch botanist Carl Thunberg in 1772 (Joubert, 1996). Generally, the leaves are oxidized or fermented to produce the distinctive reddish-brown colour, but unfermented "green" rooibos is also produced. It has been found that fermentation of rooibos leaves decrease the aspalathin and nothofagin content.

In South Africa, rooibos tea has been used as a traditional folk medicine to alleviate the symptoms of various diseases associated with digestion, respiration,

sleep and the skin (Joubert et al., 2008). Currently, the incorporation of rooibos extract into skin care products and natural remedies has become quite prominent. The potential health effects of products containing rooibos extracts or their phenolic constituents has been attributed to the biological properties of rooibos investigated in various cell culture and human models (Joubert and de Beer, 2011; Joubert et al., 2008; Lee and Jang, 2004; Magcwebeba, 2013; Marnewick et al., 2005, 2009, 2011; Petrova, 2009; Sissing et al., 2011; Snijman et al., 2009; Yoo et al., 2008, 2009). Rooibos extracts to scavenge radicals have been shown to inhibit lipid peroxidation and DNA damage, a bioactivity which has been linked to its radicals scavenging ability (Joubert et al., 2008; Lee and Jang, 2004). They also demonstrated that the flavonoids protect cells against the reduction of cell viability and cytotoxicity induced by H_2O_2 (Yoo et al., 2008, 2009). The induction of antioxidant enzymes are associated with the ability to protect against oxidative stress in cells (Marnewick et al., 2005, 2009, 2011; Yoo et al., 2008), an effect also demonstrated in animal models and humans. Marnewick et al. (2003) showed the reduction of oxidised glutathione (GSSG) and increased glutathione (GSH) levels, thus enhancing the antioxidant status in rat liver. In humans, an increase in rooibos consumption increased GSH as well as the GSSG/GSH ratio in plasma (Marnewick et al., 2011). The chemopreventive properties of rooibos against skin cancer development have been demonstrated in mouse models. The application of rooibos extracts inhibited tumour promotion by reducing the size and number of chemically-induced tumours on mouse skin (Marnewick et al., 2005). Petrova et al. (2009) also demonstrated the activity of rooibos extracts against UVB-induced tumour incidence and volume in mouse skin. Rooibos showed chemopreventive properties in cancer promotion in the liver of rats (Marnewick et al., 2009), which was attributed the presence of these two rare polyphenols.

2.3.3. Honeybush teas

More than 20 *Cyclopia* species grow endemically in the coastal and mountain regions of the Western and Eastern Cape region of South Africa (Joubert and deBeer, 2011), and have been used traditionally for the preparation of honeybush tea (Joubert et al., 2008), traditionally used in the Cape since the 18th century to treat

various ailments such as respiratory infections, digestive problems, dermatological problems, boosting the immune system, menopausal relief and stimulating breast-milk production. (Joubert and de Beer, 2011; Joubert et al., 2008). *Cyclopia maculata,* which is used as an herbal tea in farming communities in the Western Cape, has been shown to exhibit anti-obesity properties, as an extract inhibits the differentiation of adipocytes (Dudhia et al., 2013), while the study by Pheiffer et al. (2013) provided evidence that *C. maculata* stimulates lipolysis in adipocytes.

Marnewick et al (2005) and Petrova (2009) showed the potential honeybush to prevent skin cancer development. Consumption of aqueous extracts of *C. intermedia* reduced oxidised glutathione and increased GSH levels, thus protecting against oxidative stress in rats (Marnewick et al., 2003). According to Joubert et al. (2008) the total polyphenolic content of honeybush species is not a good indicator of total antioxidant activity the extracts possess. The antioxidant activity of honeybush extracts was lower than rooibos and *Camellia sinensis*. *C. intermedia* reduced the incidence and volume of 7,12-Dimethylbenz[α]anthracene (DMBA)- initiated and 12-O-tetra-decanoylphorbol-13-acetate (TPA)-promoted tumours (Marnewick et al., 2005). Petrova (2011) showed that mangiferin and hesperidin, exhibit protective effects against tumour promotion in mouse skin, though the compounds displayed lower activity when compared to the honeybush extract.

2.4. Conclusion

Natural compounds found in plants have shown potential as chemopreventive agents in these studies and due to their apparent safety, low toxicity and general acceptance (Katiyar, 2003; Katiyar et al., 1999b). Polyphenols are an extensively studied group of compounds which possess many health properties. Comprehensive literature exists on anti-carcinogenic properties of green and black tea and although the mechanisms involved are complex, anti-mutagenic, anti-inflammatory and anti-proliferative activities have been proposed to be involved. The South African herbal teas rooibos and honeybush have been known for their health promoting properties and although there are some recent studies that have substantiated their protective

properties against disease development, more scientific evidence is needed to validate these claims.

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Chapter 3

COMPARATIVE ANTIOXIDANT PROPERTIES OF *CYCLOPIA MACULATA, CAMELLIA SINENSIS*, *ASPALATHUS LINEARIS* AND *C. SUBTERNATA*

ABSTRACT

Polyphenols are present in a variety of dietary sources such as fruit, vegetables and tea and have been associated with the prevention of disease. *Camellia sinensis* (green tea) is rich in flavanols, of which the most prevalent are the catechins epicatechin (EC), epicatechin gallate (ECG), epigallocatechin (EGC), and epigallocatechin gallate (EGCG). *Aspalathus linearis* (rooibos) is considered to be rich in dihydrochalcones (DHC), the most prevalent of which are aspalathin and nothofagin. *Cyclopia* spp. (honeybush) differs from green tea and rooibos and includes the xanthones (Fig. 3.4) such as mangiferin and iso-mangiferin and flavanones, such as hesperidin. The preparation, chemical characterization and antioxidant properties of aqueous extracts of unfermented rooibos and two honeybush species, *C. subternata* and *C. maculata*, were described utilising green tea as benchmark. The effects of the major polyphenolic constituents of green tea, rooibos, *C. subternata* and *C. maculata* were also critically assessed in relation to their antioxidant properties. Green tea exhibited the highest antioxidant activity when utilising the 2,2-azinobis-(3-ethyl benzothiazoline-6-sulfonic acid) (ABTS) and 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assays while a similar activity was recorded in the ferric reducing antioxidant potential (FRAP), oxygen radical absorbance (ORAC) and lipid peroxidation assays. The honeybush herbal teas exhibited a lower antioxidant activity with *C. maculata* displaying the lowest antioxidant activity despite having higher levels of the honeybush polyphenols, mangiferin and iso-mangiferin. Different parameters such as the redox potential and interaction with Fe(II) appear to important factors in determining the antioxidant activity of the different extracts.

3.1. Introduction

Polyphenols have gained considerable popularity since they possess potent antioxidant properties that are associated with the prevention of disease (Afaq, 2011; Dai and Mumper, 2010; Pandey and Rizvi, 2009; Rahman et al., 2006). They are present in a variety of dietary sources such as fruit, vegetables and tea (Pandey and Rizvi, 2009). Flavonoids are subgroups of polyphenols most abundantly present in beverages produced from plant material, which include herbal infusions (Heim et al., 2002; Tsao, 2010). Flavonoids have a common C6–C3–C6 structure (Fig. 3.1) consisting of two aromatic rings (A and B) linked through a three carbon chain, usually organized as an oxygenated heterocyclic ring structure. This group of polyphenols can be divided into further sub-groups, due to changes and substitutions in and on the ring structures, which include anthocyanins, flavones, isoflavones, flavanones, flavonols and flavanols (Heim et al., 2002; Tsao, 2010).

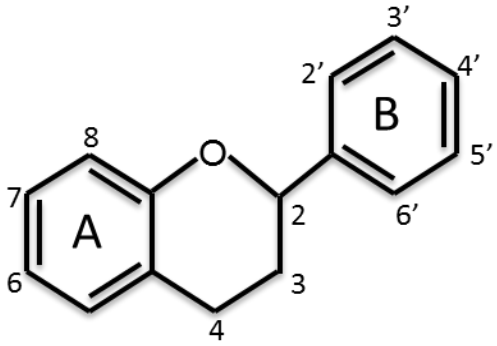


Fig. 3.1 Flavonoid backbone structure and numbering pattern common for flavonoids. (Pandey and Rizvi, 2009)

Camellia sinensis (green tea) is rich in flavanols, of which the most prevalent are catechin, epicatechin (EC), epicatechin gallate (ECG), epigallocatechin (EGC), and epigallocatechin gallate (EGCG) (Fig. 3.2) (Cabrera et al., 2006; Cavet et al., 2011). These compounds exhibit antioxidant effects by inhibiting reactive oxygen species (ROS) formation and scavenging free radicals (Tipoe et al., 2007). The beneficial effects of green tea have been studied extensively with the protective effects attributed to the green tea catechins (Cabrera et al., 2006; Cavet et al., 2011; Katiyar et al., 1999; Lambert and Elias, 2010; Suzuki et al., 2012; Tipoe et al., 2007). According to Tipoe et al. (2007), EGCG reduces the expression and production of the inflammatory mediator, COX-2 and the nitrogen radical producing inducible nitric oxide synthase (iNOS). The antioxidant properties seem to play a protective role against the adverse effects of reactive oxygen and nitrogen species produced by a variety of normal functions in the human body (Cavet et al., 2011; Rice-Evans, 1999). As a buildup of these reactive species is also observed during chronic inflammation, it is believed that the antioxidant properties of the polyphenols may also play an important anti-inflammatory role (Wu et al., 2014).

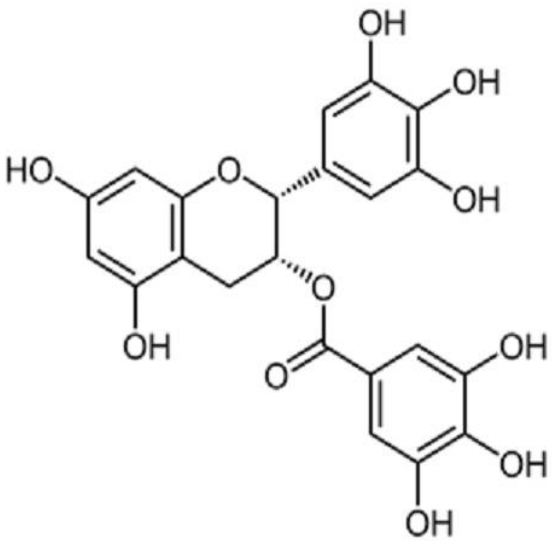


Fig 3.2 The chemical structure of the EGCG, the major polyphenolic compound present in green tea

Aspalathus linearis (rooibos) is considered to be rich in dihydrochalcones (DHC), the most prevalent of which are the C–C linked dihydrochalcone glucoside, aspalathin (Fig. 3.3) and the 3-dehydroxy dihydrochalcone glucoside, nothofagin (Joubert et al., 2008a). Both are very rare and to date aspalathin has been identified in rooibos only. Rooibos showed chemopreventive properties in cancer promotion in the liver of rats (Marnewick et al., 2009), which was attributed the presence of polyphenols. Rooibos has strong antimutagenic effects (Marnewick et al., 2000; Van der Merwe et al., 2006; Snijman et al., 2007), significantly reduced the number of papilloma's in the oesophagus (Sissing et al., 2011) and inhibited the promotion of skin tumours (Marnewick et al., 2005)

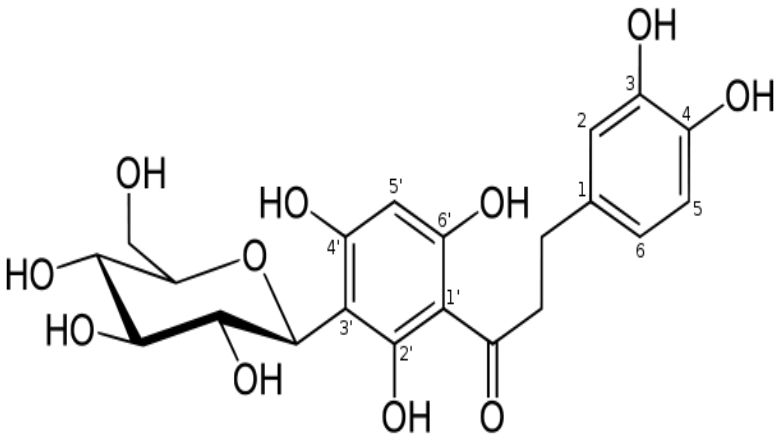


Fig 3.3 The chemical structure of aspalathin, the major polyphenolic component present in rooibos.

The polyphenolic profile of *Cyclopia* spp. (honeybush) differs from green tea and rooibos and includes xanthones such as mangiferin (Fig. 3.4) and iso-mangiferin, and flavanones, such as hesperidin. Although reports are limited, the honeybush species, *C. maculata,* which is used as a herbal tea in farming communities in the Western Cape, has been shown in two recent studies to have anti-obesity properties (Dudhia et al., 2013; Pheiffer et al., 2013).

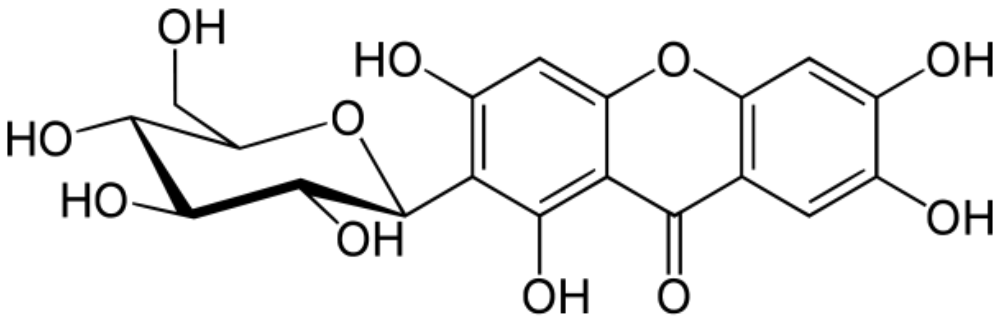


Fig 3.4 The chemical structure of mangiferin, the major polyphenolic component present in *Cyclopia spp.*

The present chapter describes the preparation, chemical characterization and antioxidant properties of aqueous extracts of green tea, rooibos and two honeybush species, *C. subternata* and *C. maculata.* The aqueous extracts obtained were used throughout the current study.

3.2. Material and Methods

3.2.1. Chemicals

2,2'-azinobis (3-ethyl-benzothizoline-6-sulfonic acid) (ABTS), thiobarbituric acid (TBA), 6-hydroxy-2, 5, 7, 8-tetramethyl-chroman-2-carboxylic acid (Trolox), 2, 4, 5-tri (2-pyridyl)-S-triazine (TPTZ), gallic acid and (+)-catechin (>96%) were obtained from Sigma-Aldrich, (Johannesburg, South Africa). Folin-Ciocalteau reagent, *p*-dimethylaminocinnamaldehyde (DAC), 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) and all other analytical reagents used were purchased from Merck, (Darmstadt, Germany).

3.2.2. Plant material.

Green tea, imported from China, was a gift from Vital Health Foods (Kuilsriver, South Africa). Unfermented rooibos was obtained from Rooibos Ltd (Clanwilliam, South Africa), while unfermented honeybush (*C. subternata and C. maculata*) was provided by the Agricultural Research Council, Infruitec-Nietvoorbij (Stellenbosch, South Africa).

3.2.3. Preparation of extracts.

Aqueous extracts of the different plant materials were prepared by steeping 100 g plant material in 1L freshly boiled deionised water for 30 min. Extracts were filtered through a single layered cheese cloth to remove the majority of the plant material, followed by stepwise filtration using Whatman No. 4 and No.1 filters. The filtrates were freeze-dried and lyophilised material stored desiccated in amber vials at room temperature.

3.2.4. Chemical characterisation

Total polyphenol content: Total polyphenolic content of the samples was determined according to the method of Singleton and Rossi (1965) with minor modifications to accommodate the microplate format. A standard curve was generated using gallic acid, using six concentrations ranging from 10 to 100 mg/L) and the dried extract, dissolved in distilled water, was assayed using five concentrations ranging from 0.05 to 0.25 g/ml. Samples, 20 µl, were added to 100 µL Folin-Coicalteau reagent (10X) and 80 µL 7.5% Na_2CO_3 (m/v) and incubated at 30°C for 2 hrs. Total polyphenol content was determined using a Biotek Synergy HT microplate reader (Winooski, Vermont, USA) and expressed as mg gallic acid equivalents/100 mg extract.

Flavanol and proanthocyanidin content: Quantification of flavanol and their oligomers, the proanthocyanidins, was carried out according to the method of McMurrough and McDowell (1978) with minor modifications to accommodate the microplate format. A standard curve was generated using catechin prepared in methanol (500 mg/l) using six concentrations ranging from 2 to 25 mg/ml. Dried extracts were dissolved in distilled water and assayed using four concentrations ranging from 0.05 to 1 mg/ml. The *p*-dimethylaminocinnamaldehyde (DAC) reagent (500 mg/l) was prepared immediately before use in methanol: hydrochloric acid, 3:1 (v/v). The catechin standards and the tea and herbal tea extracts, 35 ul, were added to 175 µl DAC reagent and incubated at 30°C for 2 hrs. The absorbance was determined spectrophotometrically at 640 nm on a Biotek Synergy HT microplate reader (Winooski, Vermont, USA) and the flavanol and proanthocyanidin (flavanol/proanthocyanidins) content was determined using the generated standard curve and expressed as mg catechin equivalents/100 mg extract.

High performance liquid chromatography (HPLC) analyses: The monomeric polyphenolic content were analysed at the Agricultural Research Council, Infruitec-Nietvoorbij (Stellenbosch, South Africa) according to Lin et al. (2008) for green tea, Beelders et al. (2012) for rooibos and De Beer and Joubert (2010) for the honeybush extracts. The content of the individual polyphenolic compounds were expressed as µg/mg extract.

3.2.5. Antioxidant properties

All antioxidant assays except the TBARS assay were performed on a Biotek Synergy HT microplate reader (Winooski, Vermont, USA). A 1 mg/ml solution of each extract was prepared in deionised water and aliquots stored at -20°C until needed.

2,2-Diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$) radical scavenging assay: The DPPH$^{\bullet}$ radical scavenging assay determines the ability of the antioxidant to scavenge free radicals

(Brand-Williams et al., 1995). A DPPH$^{\bullet}$ solution was prepared in methanol (0.1 mg/ml) and sonicated for 5 min. A standard curve was generated using Trolox at 6 concentrations ranging from 50 to 400 µM. The dried extract, dissolved in methanol, was assayed using five concentrations ranging from 0.08 to 0.16 mg/ml. DPPH$^{\bullet}$ reagent, 270 µl, was added to 30 µl extract samples and Trolox standards and incubated for 110 min at room temperature in the dark. Absorbance was determined spectrophotometrically at 515 nm and data were expressed as µmol Trolox equivalents/mg extract using the generated standard curve.

Ferric reducing antioxidant potential (FRAP): FRAP determines the ability of the samples to reduce iron (III) to iron (II) and the assay was conducted according to the method of Benzie & Strain (1996), modified for the microplate format. The FRAP reagent was prepared by using 300 mM acetate buffer, pH 3.6, 10 mM TPTZ and 20 mM $FeCl_3$ in a 10:1:1 ratio. The ratio provided a final concentration of 250 mM acetate buffer, 0.83 mM TPTZ and 1.67 mM $FeCl_3$. A standard curve was generated using Trolox, at concentrations ranging from 50 to 500 µM. The FRAP reagent, 180 µL, was added to 20 µL extracts samples or Trolox standards, incubated at 37°C for 4 min after which the absorbance was determined spectrophotometrically at 592 nm. Data were expressed as µmol Trolox equivalents/mg extract using the generated standard curve.

Oxygen radical absorbance capacity (ORAC): ORAC assays were performed according to the method described by Huang et al. (2005). Trolox was used as a standard at six concentrations ranging from 5 to 30 µM and prepared in 75 mM potassium phosphate buffer, pH 7.4. The same buffer was used to dissolve the extracts at three concentrations ranging from 5 to 7 mg/l and the reaction components, 81.6 nM fluorescein and 15mM 2,2'-azobis(2-amidinopropane) dihydro-chloride (AAPH). Extract and Trolox samples, 25 µl, were added to 150 µl fluorescein and incubated at 37°C for 10 min. Thereafter, 25 ul AAPH was added to the reaction mixture and readings taken at

485 nm (excitation) and 530 nm (emission) every minute for 35 min. Antioxidant activity was determined using the generated standard curve and expressed as µmol Trolox equivalents /g extract

ABTS assay: The radical scavenging activity of the extracts were determined using the cation 2,2-azinobis –(3-ethyl benzothiazoline-6-sulfonic acid) (ABTS) decolourisation assay (Re et al., 1999). The green tea and herbal tea extracts were prepared (from 1 mg/mL stock) in deionised water. Trolox was used as a standard at six concentrations ranging from 50 to 300 µM and prepared in ethanol. The ABTS reagent was prepared by adding 88 µL 140 nM $K_2S_2O_8$ to 5 mL 7nM ABTS solution and storing the mixture at room temperature in the dark for 12-16 hrs. The samples and Trolox standard dilutions (20 µl) were added to 180 µL ABTS reagent and incubated at 30 °C for 4 min. Absorbance was measured at 734 nm. Data were expressed as µmol TE/mg extract utilising a Trolox standard curve.

Lipid peroxidation:

(i) Preparation of microsomes: Microsomes were prepared from male Fischer 344 rats. Rat livers were homogenized in 10 mM potassium phosphate buffer, pH 7.4 containing 0.15 M KCl, centrifuged at 9000 x g and the supernatant, stored at -80°C until further purification. A microsomal fraction was isolated using Sepharose 2B column as previously described by Gelderblom et al. (1984). Protein determination was carried out using the Pierce (BCA) protein assay (Smith et al., 1985). Aliquots (2mg/ml) were stored at -80°C until further use.

(ii) TBARS assay: Inhibition of lipid peroxidation in rat liver microsomes by the extracts was determined by the method described by Snijman et al. (2009). The final incubation mixture, 1 ml, contained 100 µl green tea and the herbal tea extracts dissolved in DMSO, 200 µl 0.01 M potassium phosphate buffer, pH 7.4, and 500 µl microsomal preparation (1 mg protein/ml). Lipid peroxidation was induced by the addition of 200 µl 2.5 mM $FeSO_4$. Positive control incubations

were conducted in the absence of the extracts. Microsomal control incubations were conducted in the absence of both extract and $FeSO_4$. Samples were incubated for 1 hr at 37°C and the reaction was terminated with the addition of cold 2 ml 10% trichloroacetic acid (TCA) (m/v) solution containing 0.01% butylated hydroxytoluene (BHT) (m/v) and 1 mM ethylenediaminetetraacetic acid (EDTA). Following centrifugation at 2000 x rpm for 15min (Megafuge 1.0R, Heraeus, Hanau, Germany), 2 ml supernatant was mixed with 2 ml 0.67% thiobarbituric acid (TBA) (m/v) solution, incubated for 20 min at 90°C and the TBARS measured at 532 nm (Kontron Uvikon 923 Double Beam Spectrophotometer, BioTek, Winooski, Vermont, USA). The percentage inhibition was calculated as follows:

$$\frac{(A_{sample} - A_{control})}{A_{positive\ control}} \times 100$$

The concentrations of each extract required to achieve 50% inhibition (IC_{50}) were calculated using GraphPad Software Prism Version 5.00 for Windows (GraphPad Software, La Jolla, California, USA).

3.2.6. Statistical analysis

An analysis of variance (ANOVA) was used to test for significant group effects. For unbalanced data Tukey-Cramer adjustments were automatically made. Statistical significance was set at $P<0.05$.

3.3. Results

3.3.1. Chemical analysis

Yield: Green tea yielded the highest amount of extract, followed by *C. subternata.* Rooibos and *C. maculata* yielded the lowest levels of extract (Table 3.1).

Total polyphenol content: The extracts from green tea and rooibos displayed the highest levels of total polyphenols that did not differ significantly from each other. *Cyclopia subternata* and *C. maculata* extracts showed the lowest total polyphenols.

Table 3.2 Yield and phenolic content of aqueous extracts of green tea, rooibos and honeybush herbal plant material

	Camellia sinensis	***Aspalathus linearis***	***Cyclopia subternata***	***Cyclopia maculata***
Extract yield (%)*	26.5	16.3	22.97	17.9
Total polyphenols	26.4±0.74[a]	26.8±0.9.8[a]	22.23±1.1[b]	16.9±0.3.5[c]
Flavanols/proanthocyanidins (mg CE/100mg extract)	13.05±0.80[a]	2.00±0.33[b]	1.28±0.17[c]	0.83±0.08[d]

Values represent means ± standard deviations of triplicate replications. Statistical analyses included Wilcoxon Rank Sum Test to test for differences between the extracts. Significant differences are indicated with lower case letters in superscript (P<0.05). Abbreviations: GAE – gallic acid equivalents; CE – catechin equivalents. *Extract yields obtained from 100 g of dry plant material extracted once.

Flavanol/proanthocyanidin content: The green tea extract contained the highest flavanol levels which was approximately 6-fold greater than the rooibos extract containing the second highest level (Table 3.1). The honeybush extracts displayed significantly (P<0.05) lower levels with *C. maculata* containing the lowest levels.

HPLC analysis of monomeric polyphenols: EGCG was the most common flavanol present in the green tea extract, followed by the alkaloid, caffeine. EGC was also present in relative high quantities, but much lower levels of the other catechins were also detected, amongst which were, in decreasing order, ECG, EC and (+)-catechin (Table 3.2).

Table 3.3 Content of monomeric compounds in an aqueous extract prepared from green tea

	Compound	µg compound/mg extract
Flavanols	EGCG	42.16±0.09
	EGC	30.34±1.97
	ECG	8.19±0.20
	EC	7.84±2.38
	(+)-Catechin	0.81±0.20
Alkaloid	Caffeine	41.09±0.01

Values represent means ± standard deviations (STD) of three analyses. Abbreviations: EGCG - epigallocatechin gallate; EGC - epigallocatechin; ECG - epicatechin gallate; EC – epicatechin

The dihydrochalcones (DHC), aspalathin and nothofagin, were the major polyphenols in the rooibos extract followed by the flavones, isoorientin, orientin, isovitexin and vitexin. The flavonols present in the rooibos extract at levels that could be quantified, were quercetin-3-*O*- robionobioside (QROB), rutin, isoquercitrin and hyperoside (Table 3.3).

Table 3.3 Content of monomeric compounds in aqueous extract prepared from rooibos

	Compound	µg compound/mg extract
Dihydrochalcones	Aspalathin	96.72
	Nothofagin	13.77
Flavones	Isoorientin	11.11
	Orientin	6.84
	Vitexin	1.41
	Isovitexin	1.91
Flavonols	Rutin	3.90
	Hyperoside	2.18
	Isoquercitin	2.81
	Quercetin-3-*O*-robinobioside	8.32

Values represent a single replication of analysis.

The major honeybush polyphenols in both extracts were the xanthones, mangiferin and isomangiferin, followed by the flavanone, hesperidin. *Cyclopia maculata* contained higher levels of mangiferin (2.2-fold) and isomangiferin (2.2-fold), while hesperidin levels were also slightly higher than in *C. subternata.* However, *C. subternata* contained higher levels of the other flavanones, eriocitrin and eriodictyol-glucoside (EDG). While eriocitrin and the flavones, luteolin, were present at similar levels, *C. subternata* contained significantly higher levels of eriodictyol-glucoside (EDG), together with the flavone, scolymoside which was not detected in *C. maculata* (Table 3.4).

Table 3.4 Content of monomeric compounds in aqueous extracts prepared from *C. subternata* and *C. maculata*

	Compound	µg compound/mg extract	
		Cyclopia subternata	***Cyclopia maculata***
Xanthones	Mangiferin	27.01	61.88
	Isomangiferin	9.96	17.62
Flavanones	Eriocitrin	3.17	2.47
	Hesperidin	6.96	9.40
	EDG	3.39	0.41
Flavones	Luteolin	0.12	0.15
	Scolymoside	2.50	nd

Values represent a single replication of analysis. Abbreviations: EDG - eriodictyol-glucoside.

3.3.2. Antioxidant properties

ABTS assay: The green tea extract displayed significantly higher ($P<0.05$) radical scavenging activity than rooibos extract. Both honeybush species were significantly less ($P<0.05$) active than green tea and rooibos, while *C. subternata* was significantly ($P<0.05$) more active than *C. maculata*.

DPPH assay: Green tea exhibited the highest activity in its ability to scavenge this radical, followed by rooibos and *C. subternata with C. maculata* displaying the lowest activity. The radical scavenging activity of all extracts differed significantly ($P<0.05$) from each other.

ORAC assay: The activity of the extracts in scavenging peroxyl radicals was similar for green tea and rooibos, which were significantly more active than the honeybush species. *Cyclopia maculata* again displayed the lowest activity.

FRAP assay: The ferric iron reducing potential of the green tea and rooibos extracts did not differ significantly from each other. The honeybush extracts exhibited significantly lower activity, with the lowest activity obtained by *C. maculata*.

Table 3.5 Comparative antioxidant activity analyses of aqueous extracts of green tea, rooibos and honeybush plant material.

	Camellia sinensis	***Aspalathus linearis***	***Cyclopia subternata***	***Cyclopia maculata***
ABTS (µmol TE/mg extract)	4.17±0.26[a]	3.32±0.12[b]	2.65±0.16[c]	2.18±0.08[d]
DPPH (µmol TE/mg extract)	2.5±0.25[a]	1.92±0.06[b]	1.47±0.02[c]	1.15±0.03[d]
ORAC (µmol TE/mg extract)	4.13±0.26[a]	4.17±0.25[a]	3.49±0.25[b]	2.94±0.27[c]
FRAP (µmol TE/mg extract)	1.76±0.20[a]	1.51±0.03[a]	1.08±0.02[b]	0.91±0.04[c]
Lipid peroxidation:				
TBARS (IC_{50} - mg/ml)	0.09±0.03[a]	0.15±0.04[a]	0.32±0.03[b]	0.27±0.09[b]

Values represent means ± STD of at least three replications. Statistical analyses included One-Way ANOVA using generalised linear models and Wilcoxon Rank Sum test to test for differences between the extracts. Statistically significant differences are indicated with differing lower case letters in superscript. P<0.05 was considered significant. Abbreviations: TE – Trolox equivalents; IC_{50} – concentration yielding 50% inhibition. FRAP - Ferric reducing antioxidant potential; ABTS - 2,2'-azinobis (3-ethyl-benzthiazoline-6-sulphonic acid) TBARS - Thiobarbituric acid reacting substances; ORAC – Oxygen radical scavenging capacity.

Lipid peroxidation*: TBARS assay:* Green tea and rooibos displayed the strongest inhibition of iron-induced lipid peroxidation, however, there were no significant difference between these two extracts. There was no significant difference between the activities of

C. subternata and *C. maculata,* and they were significantly (P<0.05) less active than green tea and rooibos (Table 3.5).

3.4. Discussion

Investigations into the chemical and biological properties of *Cyclopia* species have mainly focussed on *C. intermedia* and, to a certain extent, on *C. subternata* and *C. genistoides* (Joubert et al., 2003, 2008a, 2008b). However, as a result of the increasing local and international demand for honeybush the commercial development of other *Cyclopia* species is of interest. Among these, *C. maculata* attracts attention regarding its role as an anti-obesity nutraceutical. The current study highlighted the antioxidant activity and the polyphenolic composition of aqueous extracts of *C. maculata* and *C. subternata* using rooibos and green tea as benchmark.

Previous studies indicated that rooibos contains significantly higher total polyphenol levels than green tea while the levels for the *C. subternata* were reported to be similar to rooibos (Magcwebeba, 2013; Sissing et al., 2011). Comparable levels of total polyphenols in green tea, unfermented rooibos and *C. subternata* extracts were also reported by Van der Merwe (2005). Variations in experimental methods of plant material extraction utilised and the type of plant material, related to processing of plant material and growth conditions, are likely responsible for the inter-experimental differences. As expected the green tea extract used in the present study contains the highest levels of flavanols, mainly comprised by the monomeric tea catechins (Fig. 3.2), which was confirmed by other studies when comparing to rooibos and honeybush herbal teas (Joubert et al., 2008a; Magcwebeba, 2013; van der Merwe, 2005; Sissing et al., 2011). As expected, aspalathin also constituted the major rooibos flavonoid in the current extract (9.7%), a level of which could reach up to 12% depending on the source of unfermented plant material (Beelders et al., 2012). Mangiferin, isomangiferin and hesperidin constituted the major polyphenols in honeybush with *C. maculata* exhibiting levels comparable to *C. intermedia* (Joubert et al., 2003). Aqueous extracts of unfermented *C. subternata* and *C. maculata* prepared for a previous study also

displayed similar levels of mangiferin, isomangiferin and hesperidin to the current extracts (Dudhia et al., 2013). Of interest is that, in the current study, the mangiferin and isomangiferin levels detected in *C. maculata* were 2-fold higher when compared to *C. subternata*. This species could therefore be a valuable commercial source for mangiferin, together with the other two xanthone-rich species, *C. longifolia* and *C. genistoides,* which was also shown to have high levels of mangiferin (Joubert et al., 2003; Magcwebeba, 2013).

The overall low antioxidant activity of the *Cyclopia spp.*, when compared to green tea and rooibos, has also been reported, which was aligned with the general notion that the major honeybush polyphenols exhibited a lower activity (Joubert et al., 2008a). The major green tea and rooibos flavonoids, EGCG, and aspalathin, respectively were the most active radical scavengers, in the ABTS assay, among a large panel of rooibos polyphenols tested previously (Snijman et al., 2009). It is possible that, being present at significantly higher levels, these compounds are the main contributors to the antioxidant activity when considering the high electron donating capacity of green tea and rooibos extracts in the ABTS and DPPH radical scavenging assays. Apart from that, the other major green tea catechins, EC, EGC and ECG and rooibos flavones, orientin, isoorientin and the flavonol, rutin, will also be contributing factors. The aqueous extracts of the two honeybush species, are significantly less active when considering their radical donating ability, although it has been reported to be similar to aqueous extracts of green tea and rooibos (Magcwebeba, 2013). In this regard, the extracts of the two *Cyclopia* species exhibited significantly lower total polyphenol levels when compared to green tea and rooibos with *C. maculata*, exhibiting the lowest antioxidant activity, having the lowest total polyphenol levels. Of interest is that the *C. maculata* extract, having higher levels of xanthones (mangiferin and isomangiferin) and hesperidin exhibited a weaker radical scavenging response when compared to *C. subternata*.

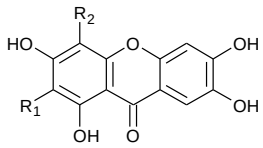
The antioxidant properties of the different extracts were further elucidated in terms of their ability to scavenge a peroxyl radical as assayed by the ORAC assay. Green tea and rooibos exhibited similar peroxyl scavenging activities, being more active than the

honeybush species. The hydrogen donating ability of rooibos could be attributed to the structural characteristics of the rooibos flavones, orientin, iso-orientin and the flavonols including rutin and isoquercitrin. The presence of the unsaturated 2, 3 bond and the 4-oxo function in the C ring is involved in radical stabilisation (Rice-Evans et al., 1996). Regarding the flavanols in green tea extracts, epicatechin exhibits a 7-fold stronger ORAC activity than EGCG, suggested to be attributable to the difference in number and position of OH-groups (Roy et al., 2010). It was also suggested that OH substitution at the 3' position in pyrogallol moieties contributes to the lower ORAC value of EGC and EGCG compared to EC and ECG without a hydroxyl moiety at C3' (Fig 3.5). In addition it was also hypothesized that flavanols, exhibiting a lower ORAC activity as compared to the activity utilising the DPPH assay (Roy et al., 2010), are powerful pro-oxidants when compared to substances showing a higher ORAC activity. In this regard the green tea extract exhibited the highest antioxidant activity in the DPPH and the ABTS assays when compared to rooibos which could be ascribed to the high EGCG and EGC to EC and ECG ratios of approximately 5 and 4, respectively.

The iron (III) reducing ability of the extracts was determined by the FRAP assay, with green tea and rooibos extracts exhibiting similar activities. The honeybush extracts exhibited significantly lower activity, with *C. maculata* having the lowest activity. Previous studies have also reported that the activity of green tea and rooibos were very similar (Joubert et al., 2008a; Magcwebeba, 2013), although slight differences were detected which could be related to variations in the polyphenolic composition. The low activity exhibited by the honeybush extracts, specifically that of *C. maculata*, may be attributed to the high levels of mangiferin. It has been reported that mangiferin exhibits a stabilizing effect on iron through the chelation of iron (III), which prohibits the Fenton reaction (Perron and Brumaghim, 2009).

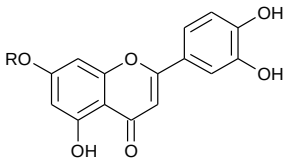
The iron chelating properties also find application in the inhibition of lipid peroxidation by the different extracts as iron acts as an inducer of oxidation. Green tea and rooibos showed the strongest ability to inhibit the end product of lipid peroxidation, malondialdehyde, displaying lower IC_{50} values than both honeybush species.

Honeybush polyphenols



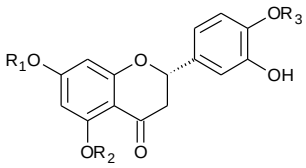
Xanthones

Mangiferin: R_1=*C*-β-D-glucopyranosyl, R_2=H
Isomangiferin: R_1=H, R_2=*C*-β-D-glucopyranosyl



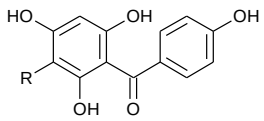
Flavones

Luteolin: R= H
Scolymoside: R=rutinosyl



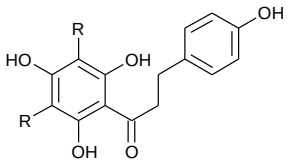
Flavanones

Hesperidin: R_1=rutinosyl, R_2 =H, R_3=CH_3
Eriocitrin: R_1=rutinosyl, R_2, R_3=H
Eriodictyol glucoside: R_1 or R_2=glucosyl, R_3=H



Benzophenone

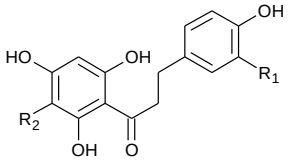
Iriflophenone-3-*C*-β-glucoside:
R=*C*-β-D-glucopyranosyl



Dihydrochalcone

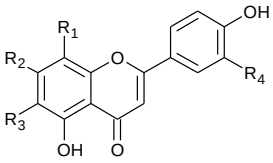
Phloretin-3',5'-di-*C*-β-glucoside:
R=*C*-β-D-glucopyranosyl

Rooibos flavonoids



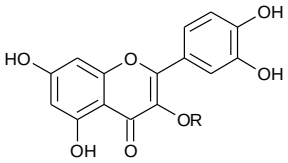
Dihydrochalcones

Aspalathin: R_1=OH, R_2=*C*-β-D-glucopyranosyl
Nothofagin: R_1=H, R_2=*C*-β-D-glucopyranosyl



Flavones

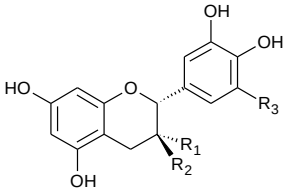
Isoorientin: R_1, R_2, R_4=OH, R_3=*C*-β-D-glucopyranosyl
Orientin: R_1=*C*-β-D-glucopyranosyl, R_2, R_3=H, R_4=OH
Vitexin: R_1=*C*-β-D-glucopyranosyl, R_2, R_3=H, R_4=H
Isovitexin: R_1, R_2, R_4=H, R_3=*C*-β-D-glucopyranosyl
Luteolin: R_1, R_3=H, R_2,R_4= OH



Flavonols

Quercetin-3-*O*-robinobioside: R=robinobiosyl
Isoquercitrin: R=β-D-glucopyranosyl
Rutin: R=rutinosyl
Hyperoside: R=galactopyranosyl

Green tea flavanols



(-)-Epigallocatechin gallate: R_1=O-gallate, R_2=H, R_3=OH
(-)-Epicatechin gallate: R_1=O-gallate, R_2,R_3=H
(-)-Epigallocatechin: R_1, R_3=OH, R_2=H
(-)-Epicatechin: R_1=OH, R_2, R_3=H
(+)-Catechin: R_1, R_3=H, R_2=OH

Fig 3.5. Diverse chemical structures of the polyphenolic compounds comprising the extracts of the green tea, unfermented rooibos and honeybush teas.(Obtained from Magcwebeba, 2013)

In a recent study it was also shown that green tea and rooibos extracts displayed similar activities in a TBARS assay (Magcwebeba, 2013). Despite the ±3-fold higher level of mangiferin and its ability to stabilise iron (III), the inhibitory effect of *C. maculata* on lipid peroxidation were similar to that of *C. subternata* imply that apart from iron chelating, other parameters such as hydrophobicity and redox potential also play a major role in determining the biological responses in a lipid environment. Polyphenols have been shown to be associated with polar phospholipid heads in the membrane environment, positioning itself at the hydrophobic/hydrophilic interphase (Snijman et al., 2009). The hydrophobicity of polyphenols determines their partitioning in the cell membranes and the resultant interactions with membrane lipids and proteins. These interactions will facilitate their solubility and hence protection against lipid peroxidation. The hydrophobic interactions and iron stabilizing effects have important implications regarding the effect of these extracts on the modulation of cell viability which are discussed in Chapters 4 and 5.

The activity of rooibos and green extracts in the electron and hydrogen donating assays when compared to that of honeybush are linked to the redox potential of the polyphenolic constituents. The highly reactive anti-oxidants EGCG of green tea, luteolin and quercetin, minor constituents of rooibos, have low redox potentials and tend to undergo auto-oxidation, and in the presence of iron also act as pro-oxidants (Moridani et al., 2002). In contrast, the honeybush polyphenols, specifically mangiferin has a far higher redox potential and not readily undergoing auto-oxidation, mainly due to its stabilizing effect of Fe(III) (Perron and Brumaghim, 2009). Detailed characterization of the extracts with respect to their chemical composition and antioxidant properties is essential for the interpretation of the studies on anti-inflammatory properties of these extracts that will be highlighted in chapters 4 and 5. As the polyphenolic constituents and antioxidant activities of the extracts prepared from different herbal plant material vary greatly, the characterization of the extracts serves as a “quality control” for the entire project. In addition, the interaction between major and minor components in the extracts is complex and need to be carefully considered to assess possible synergistic and or additive effects.

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Chapter 4

PRE-EXPOSURE *IN VITRO* MODEL FOR THE PREVENTION OF UVB-INDUCED INFLAMMATION BY ROOIBOS AND HONEYBUSH AQUEOUS EXTRACTS UTILISING HUMAN KERATINOCYTES (HaCaT)

ABSTRACT

In skin, ultraviolet B (UVB) irradiation elicits a wide range of biological effects which may lead to cancer development after chronic exposure. These effects include the induction of pro-inflammatory cytokines and the induction of apoptosis in cells. Dietary components such as tea have been shown to prevent against UVB-induced damage by modulating the inflammatory response and the proliferation of exposed cells. Green tea (*Camellia sinensis*), rooibos (*Aspalathus linearis*) and honeybush (*Cyclopia* spp.) have been demonstrated to have anti-inflammatory properties in mouse models. The aim of the current study was to determine the preventive effects of green tea, rooibos and honeybush aqueous extracts against interleukin-1α (IL-1α) accumulation in human keratinocytes. The modulation of cell growth parameter indices, cell viability and apoptosis prior to and following UVB exposure of skin keratinocytes (HaCaT) were also evaluate. All tea and herbal tea extracts reduced UVB-induced IL-1α accumulation while, depending on the concentration, maintaining cell viability without effecting apoptosis. Pre-exposure of HaCaTs to the different extracts is likely to protect against oxidative stress affected by UVB-induced irradiation. The anti-inflammatory activity of the herbal tea extracts may be due to the anti-oxidant properties of polyphenolic constituents inhibiting the multiple pathways leading to the activation of activator protein-1 (AP-1) and nuclear factor kappa B (NF-κB) pathways governing the inflammatory cytokine responses.

4.1 Introduction

Physical injury, pathogens, exposure to toxic chemicals and ultraviolet B (UVB) irradiation are external stimuli that can induce inflammation, the first response of the body to protect itself (Mak and Saunders, 2006a). The skin, the body's largest organ, plays an important role as an immune organ with keratinocytes being the principle epidermal cells and a major source of cytokines including interleukin (IL)-1, IL-6, IL-7, IL-8, IL-10, IL-12, IL-15, IL-18, and IL-20 and tumour necrosis factor-α (TNF-α) (Grone, 2002). IL-1 and TNF-α initiate a signalling cascade that induces gene expression and production of secondary mediators, which include cytokines and chemokines, growth factors, adhesion molecules, cyclo-oxygenase type 2 (COX-2), inducible nitric oxide synthase (iNOs) and other pro-inflammatory factors (Borish and Steinke, 2003; Mak and Saunders, 2006b; Steinke and Borish, 2006; Williams and Kupper, 1996). This facilitates the chemo-attraction of reactive oxygen species (ROS)-producing immune cells which aid in the repair of damaged tissue (Aggarwal et al., 2006).

Acute inflammation is rapid and self-limiting, but can become chronic if maintained over a prolonged time (Mueller, 2006). Chronic inflammation, aided by a variety of mediators of which TNF-α and IL-1α are primary mediators, is known to promote many diseases, including cancer (Aggarwal et al., 2006; Mueller, 2006). Due to its role in inflammation, UVB-induced reactive oxygen species (ROS) has led research to focus on the anti-inflammatory properties of antioxidants that occur in nature, such as plant polyphenols (Kostyuk et al., 2010). The green tea (*Camellia sinensis*) polyphenol, epigallocatechin galate (EGCG) inhibits UVB-induced immunosuppression in mouse skin by altering the production of IL-10 and IL-12 (Katiyar et al., 1999a). It also reduces the production of ROS in human skin following UVB-exposure (Katiyar et al., 1999b).

An *in vitro* inflammatory model, utilising immortalised transformed keratinocytes, HaCaT cell line was recently developed (Magcwebeba et al., 2012). It was shown that unfermented rooibos (*Aspalathus linearis*) and honeybush (*Cyclopia* spp.) herbal tea extracts exhibited indirect anti-inflammatory properties by enhancing apoptosis

and thereby protecting against the accumulation IL-1α following UVB exposure (Magcwebeba, 2013). The current study further modified the HaCaT cell model to determine the protective effects of the herbal teas against the induction of IL-1α when treated before UVB exposure. In the process of developing the pre-exposure model, the appropriate method of delivering the tea/herbal tea extracts to skin cells prior to UVB exposure was evaluated. The protection against the induction of IL-1α in the pre-exposure model was also validated by using known anti-inflammatory drugs.

4.2 Materials and methods

4.2.1 Reagents

Heat inactivated fetal bovine serum (FBS) was purchased from Invitrogen (Johannesburg, South Africa). RPMI-1640 culture medium, L-glutamine, trypsin-versene and Hank's buffered salt solution (HBSS) were obtained from Lonza (Belgium). Dulbecco's phosphate buffered saline (DPBS), dimethyl sulfoxide (DMSO), dexamethasone, ibuprofen, dimethyl sulfoxide (DMSO) and albumin bovine serum (BSA) were purchased from Sigma-Aldrich (Johannesburg, South Africa). Human recombinant interleukin-1α (IL-1α) ELISA kit was purchased from R&D systems (Minneapolis, USA), while the CellTiter-Glo®Luminescent cell viability assay and the caspase-3/7 assay kits were purchased from Promega (Madison, USA).

4.2.2 Plant material and preparation of extracts and preparation of extracts

The different plant materials and their aqueous extracts were described in detail in Chapter 3.

4.2.3 Cell culture

Spontaneously immortalised keratinocytes (HaCaT) were a gift from the Department of Human Biology of the University of Cape Town (Cape Town, South Africa). Cells were maintained in RPMI-1640 supplemented with 10% FBS (v/v) and 2 mM L-glutamine in a humidified atmosphere of 5% CO_2/95% air at 37°C. Cells

were passaged three times a week at a split ratio of 1:6. For the purposes of this study, the cells were seeded in different plates: white tissue culture (Porvair Sciences, Shepperton, UK) 96-well plates (3 x 10^4 cells per well) were used for the determination of ATP content, while 24-well plates (18 x 10^4 cells per well) were utilised for IL-1α and caspase-3 activity determinations.

4.2.3.1 Preparation of extracts for tissue culture studies.

Freeze-dried extracts were weighed and dissolved in 100% DMSO (v/v) and diluted with RPMI-1640 medium containing 0.5% FBS (v/v) to a concentration of 40 mg extract/ml containing 10% DMSO (v/v). The extracts were diluted in RPMI-1640 culture medium to a concentration of 2 mg extract/ml [0.5% DMSO (v/v)] and filter sterilised through a syringe filter (pore size 0.22 µm). The filtered solutions and RPMI-1640 medium containing 0.5% FBS (v/v) and 0.5% DMSO (v/v) were used to prepare the desired dilutions of extracts to be used in the UVB pre-exposure model. A dilution range of three concentrations was used for green tea (0.05 to 0.2 mg/ml) and *C. subternata* (0.1 to 0.4 mg/ml). A dilution range consisting of four concentrations (0.025 to 0.2 mg/ml) was used for rooibos and *C. maculata* (0.063 to 0.5 mg/ml). The extract concentrations used to screen for anti-inflammatory properties in the current investigation were based on the IC_{50} values calculated by Magcwebeba (2013) for aqueous extracts of rooibos and honeybush.

4.2.3.2 Biological endpoints measured

4.2.3.2.1 Cell viability (ATP production).

CellTiter-Glo Luminescent viability kit was used to monitor ATP content in the cells after the incubation period. Luciferase reagent, 100 µl, was added to each well containing the cells and plates were rotated for 2 min and incubated at room temperature for 10 min in the dark. The luminescent signal was determined using a Veritas™ microplate luminometer (Turner Biosystems, Sunnyvale, California, USA) and data were expressed as a percentage (%) ATP content of the control cells.

4.2.3.2.2 IL-1 α determination.

IL-1α content in cell lysates, prepared as described in section 4.2.4, was determined with an IL-1α ELISA kit (R&D systems, Minneapolis, USA) according to the manufacturer's instructions. A standard curve was generated using human recombinant IL-1α prepared in 1% BSA in DPBS (w/v) using concentrations ranging from 7.8 to 500 pg/ml. Absorbance was measured at 450 nm with a Synergy 2 Multi-Mode Microplate Reader (BioTek®, Winooski, Vermont, USA) and data were analysed using the standard curve generated from Gen5™ Data Analysis Software (version 2 for Windows). Intracellular IL-1α was expressed as pg/ml of cell lysate, normalised against ATP content relative to the control treatment as a surrogate measure for cell number and as IL-1α fold increase of the untreated control (samples not irradiated). The following formulas were used:

$$\text{Normalised IL-1}\alpha = \frac{\text{IL-1}\alpha \text{ sample in pg/ml}}{\%\ \text{Cell viability}} \times 100$$

$$\text{IL-1}\alpha \text{ Fold Increase} = \frac{\text{Normalised IL-1}\alpha \text{ (sample)}}{\text{Normalised IL-1}\alpha \text{ (control)}}$$

4.2.3.2.3 Determination of apoptosis

Cell lysates, 20 µl, were transferred into a white solid 96-well assay microplate and incubated with 20 µl caspase 3/7 reagent in the dark at room temperature for 1 hr. Luminescence was measured using a Veritas™ microplate luminometer and the data expressed as fold increase compared to the control, representing the caspase-3 activity in cells. The fold increase was also normalised against ATP content relative to the control as a surrogate measure for cell number. The following formulas were used:

$$\text{Caspase Fold Increase} = \frac{\text{RLU (sample)}}{\text{RLU (negative control)}}$$

$$\text{Normalised caspase Fold Increase} = \frac{\text{Caspase Fold Increase}}{\text{\% cell viability}} \times 100$$

4.2.4 Development of pre-exposure model: Methods of pre-exposure

In order to achieve a similar increase of intracellular IL-1α content following UV exposure as in the previously published post-exposure model (Magcwebeba et al., 2012), two different methods of delivering extracts and drugs of interest prior to UV exposure were tested. HaCaT cells were seeded in RPMI-1640 medium containing 10% FBS as described above. Plates were incubated at 37°C for 6 hrs to allow cells to attach, thereafter (i) the cells were left unchanged (UVB post-exposure model), (ii) an equal volume of medium containing 0.5% FBS was added to the wells doubling the volume in the well or (iii) the medium was replaced with fresh medium containing 0.5%FBS (v/v) and 0.5% DMSO (v/v). Cells were incubated for an additional 18 hrs. After removing the cultured medium, cells were exposed to 80 mJ/cm^2 of UVB light in 600 µl DPBS, pH 7.4 without the plastic lid, using the UVIlink UV crosslinker (UVitec limited, UK) fitted with six 8 Watt UV tubes emitting at a wavelength of 302 nm. Immediately after irradiation, the DPBS was replaced with fresh RPMI-1640 medium containing FBS (v/v) and 0.5% DMSO (v/v) and cells incubated for 24 hrs. At the end of the experiment, cells were lysed by adding 0.5% Triton-X-100 (v/v) in DPBS, pH 7.4 and stored at -80°C for intracellular IL-1α determination. The ATP content was determined separately as described in section 4.2.3.2.1.

4.2.4.1 Validation of the pre-exposure model utilising anti-inflammatory drugs

HaCaT cells were seeded as described above and dexamethasone and ibuprofen, final concentrations varying between 0.31 mM to 1.25 mM, were added to the cells 18 hrs prior to UV exposure as indicated for control [treatment (ii) as described above] in the medium containing 0.5% FBS and 0.5% DMSO After the 18 hour incubation, cells were irradiated as described above and fresh RPMI-1640 medium

containing 0.5% FBS (v/v) and 0.5 % DMSO (v/v) was added for a further 24 hrs incubation. Thereafter, cells were washed once with 600 µl DPBS and lysed with 900 µl 0.5% TritonX-100 (v/v) in DPBS, pH 7.4, and stored at -80°C for intracellular IL-1α determination. The ATP content was determined separately as described in section 4.2.3.2.1.

4.2.4.2 Screening of plant extracts for potential anti-inflammatory properties

4.2.4.2.1 Effect of extracts on cell viability, apoptosis and IL-1α accumulation before UVB exposure.

As the plant extracts, added before UV treatment, may lead to changes in the cell growth parameters, it was necessary to determine the effect of the extracts prior to UVB exposure. HaCaT cells were seeded utilising the method for the pre-exposure model as described above and different dilutions (Section 4.2.3.1) of green tea, rooibos and honeybush extracts added to the wells 6 hrs after seeding. After 18 hrs of incubation, the culture medium was removed and the cells were lysed with 0.5% TritonX-100 (v/v) in DPBS, pH 7.4 and stored at -80°C for intracellular IL-1α (section 4.2.3.2.2) and caspase-3 activity (section 4.2.3.2.3) determination. The ATP content was determined separately as described in section 4.2.3.2.1.

4.2.4.2.2 Modulation of cell growth parameters and IL-1α production by the green tea and herbal extracts in the pre-exposure UVB model

HaCaT cells were seeded utilising the method for developing the pre-exposure model as described above. The green tea and herbal tea concentrations were added in an equal volume of the culture medium to serve as the untreated UVB control. Cells were lysed with 0.5% TritonX-100 (v/v) in DPBS, pH 7.4 and stored at -80°C for intracellular IL-1α and caspase-3 activity determination while cell viability was determined separately as describe above.

4.2.5 Statistical analysis

Descriptive statistics performed on the data indicated that all variable groups were normally distributed (Kolmogorov–Smirnof Test). Where 2 main effects (both categorical) were present, statistical analyses included two-way ANOVAs testing first for a significant interaction between the two main effects. If no interaction was present, significant main effects were further investigated using the post-hoc Tukey's Studentized Range Test to test for multiple pairwise comparisons between all groups. Group differences were tested with a One-way ANOVA where only one categorical main effect was present. The post-hoc Tukey's Studentized Range Test was also used to test for multiple pairwise comparisons between all groups. As the data were unbalanced, the Tukey–Cramér adjustment was made automatically. Group differences were tested using Student's T-test when only two groups were present. Statistical significance was considered at $P < 0.05$.

4.3 Results

4.3.1 Development of the pre-exposure model

In order to ensure quantifiable IL-1α induction following UV exposure, two modes of the medium delivery were investigated, one by adding fresh medium after 6 hrs directly to the wells, and another by replacing the existing medium of cells after 6 hrs with fresh medium. Both methods were compared to the established UVB model (Magcwebeba, 2013). Compared to the control treatment, the addition of the culture medium did not significantly ($P<0.05$) affected cell viability (Table 4.1). Replacing the medium, however, resulted in a significant decrease (24%) in cell viability following UVB exposure. The increased intracellular IL-1α detected upon addition of medium was comparable to levels obtained in the established UVB exposure model. However, the cells were not responsive to IL-1α accumulation following UV exposure when the medium was replaced after 6 hrs. Adding the extracts directly to the culture medium was thus chosen as the method of the extract delivery when utilising the pre-exposure model (Fig 4.1).

Table 4.1 Effect of method of culture medium treatment on cell viability and IL-1α accumulation

	UVB-exposure Model (i)		Adding medium (ii)		Replacing medium (iii)	
	Negative Control	**Positive Control**	**Negative Control**	**Positive Control**	**Negative Control**	**Positive Control**
% ATP Content	$100.0\pm4.7^{A}_{a}$	$92.8\pm10.9^{A}_{b}$	$100.0\pm8.6^{A}_{a}$	$93.8\pm8.7^{A}_{b}$	$100.0\pm6.3^{A}_{a}$	$76.0\pm8.8^{B}_{b}$
IL-1α (pg/ml)	$48.2\pm11.8^{A}_{a}$	$131.3\pm23.0^{B}_{a}$	$38.9\pm6.0^{A}_{a}$	$87.9\pm17.8^{B}_{a}$	$44.5\pm6.5^{A}_{b}$	$32.6\pm8.7^{A}_{b}$
Normalised IL-1α (pg/ml)*	$48.2\pm11.8^{A}_{a}$	$160.0\pm27.3^{B}_{a}$	$38.9\pm6.0^{A}_{a}$	$98.9\pm20.5^{B}_{a}$	$44.5\pm6.5^{A}_{b}$	$43.7\pm11.8^{A}_{b}$
IL-1α Fold Increase	$1.0\pm0.2^{A}_{a}$	$3.0\pm0.4^{B}_{a}$	$1.0\pm0.1^{A}_{a}$	$2.4\pm0.4^{B}_{a}$	$1.0\pm0.2^{A}_{b}$	$1.0\pm0.4^{A}_{b}$

Cells were incubated at 37°C for 6 hrs, the medium was either removed and fresh medium was added or fresh medium was added. The cells were then incubated for an additional 18 hrs, cultures irradiated and incubated for an additional 24 hrs. Values represent mean ± standard deviation of at least five replications. Different uppercase letters in superscript in a row indicate differences between all negative controls and positive controls; Different lowercase letters in subscript in a row indicate the effect of UVB irradiation within (i), (ii) and (iii). *Normalised IL-1α was calculated by correcting the IL-1α according to cell viability (section 4.2.3.2.2). Significant differences was consider at $P<0.05$.

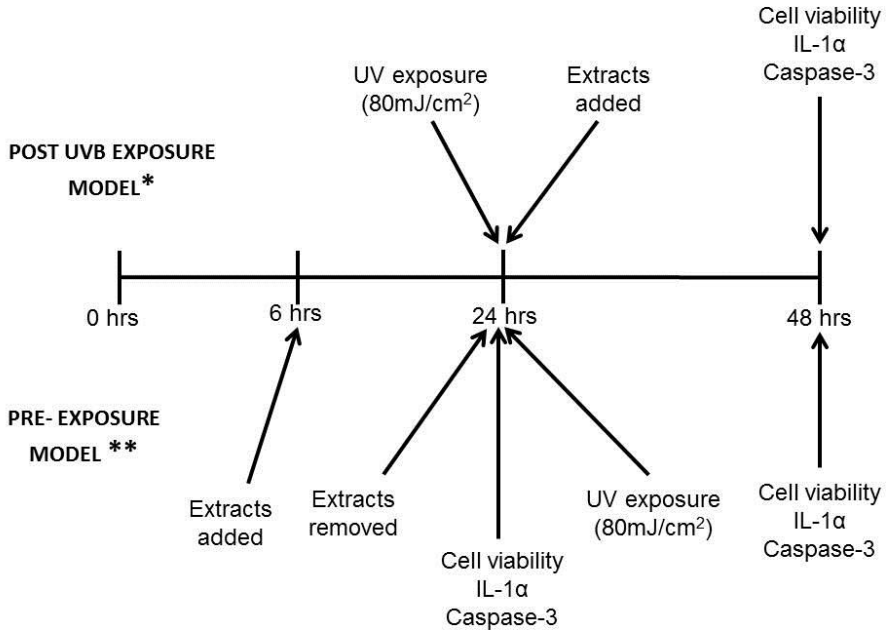

Fig. 4.1 Schematic illustration of the post-exposure model (*Magcwebeba**,** 2013) compared to the pre-exposure model (**Current investigation)

4.3.2 Pre-exposure model validation utilising anti-inflammatory drugs

Pre-exposure of the HaCaT cells to the anti-inflammatory drugs (dexamethasone and ibuprofen) did not affect cell viability compared to the positive control (Table 4.2). Ibuprofen, did not affect intracellular IL-1α at its lowest concentration (0.31 mM), while it was significantly ($P<0.05$) increased at the higher concentrations, both directly or normalised according to cell viability. Dexamethasone also significantly ($P<0.05$) increased intracellular IL-1α at all concentrations used, both directly and when normalised against cell viability.

Table 4.2 Effect of ibuprofen and dexamethasone on cell viability and IL-1α accumulation in the pre-exposure UVB model

Biological endpoints	Negative Control	Positive Control	Ibuprofen (mM)		
			0.31	0.63	1.25
% ATP content	100.0 ± 3.6^{A}	$58.0\pm6.7^{B}{}_{a}$	$59.0\pm6.8_{a}$	$55.6\pm7.5_{a}$	$46.7\pm6.0_{b}$
IL-1α (pg/ml)	24.9 ± 0.9^{A}	$49.4\pm3.5^{B}{}_{a}$	$58.0\pm12.4_{a}$	$62.7\pm7.3_{b}$	$80.9\pm5.7_{c}$
Normalised IL-1α (pg/ml)*	24.9 ± 0.9^{A}	$76.6\pm5.4^{B}{}_{a}$	$86.5\pm18.4_{a}$	$100.6\pm11.6_{b}$	$154.7\pm10.9_{c}$
IL-1α Fold Increase	1.0 ± 0.0^{A}	$3.1\pm0.2^{B}{}_{a}$	$2.9\pm0.7_{a}$	$4.0\pm0.3_{a}$	$5.7\pm0.4_{b}$
Biological endpoints	**Negative Control**	**Positive Control**	**Dexamethasone (mM)**		
			0.31	**0.63**	**1.25**
% ATP content	100.0 ± 3.6^{A}	$58.0\pm6.7^{B}{}_{a}$	$65.0\pm7.6_{a}$	$61.1\pm6.7_{a}$	$57.8\pm6.2_{a}$
IL-1α (pg/ml)	24.9 ± 0.9^{A}	$49.4\pm3.5^{B}{}_{a}$	$100.5\pm12.9_{b}$	$113.6\pm9.4_{b}$	$95.1\pm4.0_{b}$
Normalised IL-1α (pg/ml)*	24.9 ± 0.9^{A}	$76.6\pm5.4^{B}{}_{a}$	$133.3\pm49.0_{b}$	$149.9\pm50.5_{b}$	$124.3\pm36.7_{b}$
IL-1α Fold Increase	1.0 ± 0.0^{A}	$3.1\pm0.2^{B}{}_{a}$	$3.8\pm0.5_{b}$	$5.1\pm0.2_{b}$	$5.6\pm0.2_{b}$

Cells were incubated at 37°C for 6 hrs and varying concentrations of dexamethasone and ibuprofen added and the cells incubated for an additional 18 hrs. Following irradiated cultures were incubated for an additional 24 hrs. Values represent mean ± standard deviation of at least five replications. Different uppercase letters in superscript in a row indicate differences between negative control and positive control; Different lowercase letters in subscript in a row indicate differences between the positive control and concentrations of anti-inflammatory drugs. *Normalised IL-1α was calculated by correcting the IL-1α according to cell viability (section 4.2.3.2.2). Significant differences was consider at $P<0.05$.

4.3.3 Anti-inflammatory potential of extracts on cell viability, apoptosis and IL-1α accumulation in keratinocytes in pre-exposure model

4.3.3.1 Effects of different extracts on cell viability, IL-1α accumulation and apoptosis before UVB-exposure

(i) Green tea

The green tea extract displayed no effect on the viability of the HaCaT cells after 18 hrs prior to UVB exposure. It did, however, significantly ($P<0.05$) decreased intracellular IL-1α when compared to the control (Table 4.3). The highest concentration of the extract decreased the cellular IL-1α (pg/ml) content by approximately by 50 %. There were no significant differences in IL-1α content when normalised according to cell viability between the different extract concentrations. The green tea extract significantly ($P<0.05$) increased the caspase-3 activity at the highest extract concentration when compared to control (normalised data).

(ii) Rooibos

The rooibos extract did not affect cell viability significantly. The extract significantly ($P<0.05$) decreased the IL-1α content and when normalised according to cell viability at all the concentrations although no dose response effects were detected. The rooibos extract, resulted in a 1.5-fold increase in caspase-3 activity at the highest concentration (0.2 mg/ml) when considering both the direct and the normalised data (Table 4.3), while the lower concentrations displayed no effect.

Table 4.3 Effect of green tea and rooibos on cell viability, intracellular IL-1α and apoptosis of HaCaT cells before UVB exposure

Biological endpoints	Control	Green tea (mg/ml)		
		0.05	0.1	0.2
ATP content (% of control)	100.0±4.8^{A}	98.4±6.7^{A}	93.2±9.8^{A}	97.2±8.1^{A}
IL-1α (pg/ml)	59.1±7.9^{A}	30.2±6.2^{B}	27.6±7.6^{B}	26.2±7.9^{B}
Normalised IL-1α (pg/ml)*	59.1±7.9^{A}	32.6±8.3^{B}	30.5±10.2^{B}	27.4±8.9^{B}
IL-1α (Fold decrease)	1.0±0.1^{A}	0.6±0.1^{B}	0.5±0.1^{B}	0.5±0.1^{B}
Caspase-3 (Fold Increase)	1.0±0.1^{A}	1.2±0.3^{A}	1.2±0.2^{A}	1.8±0.3^{B}
Normalised Caspase-3 (Fold Increase)**	1.0±0.1^{A}	1.2±0.6^{A}	1.4±0.6^{A}	1.8±0.6^{B}

Biological endpoints	Control	Rooibos (mg/ml)			
		0.025	0.05	0.1	0.2
ATP content (% of control)	100.0±4.8^{A}	103.1±10.3^{A}	105.1±10.8^{A}	101.6±9.4^{A}	101.9±4.9^{A}
IL-1α (pg/ml)	59.1±7.9^{A}	40.5±10.6^{B}	38.7±14.1^{B}	40.4±11.3^{B}	43.3±21.7^{A}
Normalised IL-1α (pg/ml)*	59.1±7.9^{A}	39.8±15.9^{B}	40.6±10.6^{B}	41.4±13.1^{B}	42.2±21.0^{A}
IL-1α (Fold decrease)	1.0±0.1^{A}	0.8±0.2^{A}	0.8±0.2^{A}	0.8±0.2^{A}	0.6±0.3^{A}
Caspase-3 (Fold Increase)	1.0±0.1^{A}	0.9±0.2^{A}	1.0±0.4^{A}	1.1±0.4^{A}	1.5±0.2^{B}
Normalised Caspase-3 (Fold Increase)**	1.0±0.1^{A}	0.9±0.5^{A}	1.0±0.5^{A}	1.1±0.4^{A}	1.5±0.2^{B}

Cells were incubated at 37°C for 6 hrs, varying concentrations of green tea and rooibos added and the cells incubated for an additional 18 hrs. Values represent mean ± standard deviation of at least five replications. Different uppercase letters in superscript in a row indicate differences between the untreated control and cells treated with different concentrations of the extracts. *Normalised IL-1α was calculated by correcting the IL-1α according to cell viability (4.2.3.2.2);**Normalised Caspase-3 Fold Increase is the Caspase-3 fold increase normalised by using cell viability (section 4.2.3.2.3). Significant differences was consider at $P<0.05$.

Table 4.4 Effect of *C. subternata* and *C. maculata* on cell viability, intracellular IL-1α and apoptosis of HaCaT cells before UVB exposure

Biological endpoints	Control	*C. subternata* (mg/ml)		
		0.1	0.2	0.4
ATP content (% of control)	100.0±4.8^{A}	97.7±29.2^{A}	107.1±8.8^{A}	98.4±8.6^{A}
IL-1α (pg/ml)	59.1±7.9^{A}	33.9±10.7^{B}	31.6±14.3^{B}	35.6±18.3^{B}
Normalised IL-1α (pg/ml)*	59.1±7.9^{A}	34.8±15.8^{B}	34.4±14.1^{B}	37.3±18.5^{B}
IL-1α (Fold decrease)	1.0±0.1^{A}	0.6±0.4^{A}	0.6±0.3^{A}	0.6±0.3^{A}
Caspase-3 (Fold Increase)	1.0±0.1^{A}	1.1±0.4^{A}	0.9±0.2^{A}	1.1±0.3^{A}
Normalised Caspase-3 (Fold Increase)**	1.0±0.1^{A}	1.2±0.5^{A}	0.8±0.3^{A}	1.1±0.4^{A}

Biological endpoints	Control	*C. maculata* (mg/ml)			
		0.0625	0.125	0.25	0.5
ATP content (% of control)	100.0±4.8^{A}	97.7±10.8^{A}	95.5±10.4^{A}	94.5±9.3^{A}	92.8±5.7^{A}
IL-1α (pg/ml)	59.1±7.9^{A}	48.7±16.9^{A}	41.2±14.1^{A}	38.7±12.2^{B}	32.6±18.4^{B}
Normalised IL-1α (pg/ml)*	59.1±7.9^{A}	51.6±18.2^{A}	44.8±12.8^{A}	41.1±10.9^{A}	34.3±19.3^{A}
IL-1α (Fold decrease)	1.0±0.1^{A}	0.9±0.3^{A}	0.8±0.2^{A}	0.8±0.2^{A}	0.5±0.3^{B}
Caspase-3 (Fold Increase)	1.0±0.1^{A}	1.1±0.3^{A}	0.9±0.2^{A}	1.0±0.2^{A}	1.2±0.3^{A}
Normalised Caspase-3 (Fold Increase)**	1.0±0.1^{A}	1.1±0.3^{A}	1.1±0.4^{A}	1.1±0.3^{A}	1.4±0.4^{A}

Cells were incubated at 37°C for 6 hrs and varying concentrations of *C.subternata* and *C. maculata* added and the cells incubated for an additional 18 hrs. Values represent mean ± standard deviation of at least five replications. Different uppercase letters in superscript in a row indicate differences between the untreated control and cells treated with different concentrations of the extracts. *Normalised IL-1α was calculated by correcting the IL-1α according to cell viability (4.2.3.2.2);**Normalised Caspase-3 Fold Increase is the Caspase-3 fold increase normalised by using cell viability (section 4.2.3.2.3). Significant differences was consider at P<0.05.

(iii) Honeybush

The extract of *C. subternata* did not affect viability of HaCaT cells when added before UVB exposure. The extract significantly (P<0.05) reduced the IL-1α content (pg/ml) and when normalised according to cell viability, while the caspase-3 fold remained unchanged (Table 4.4). Different extract concentrations of *C. maculata* also did not affect cell viability of the keratinocytes after the 18 hrs of exposure. However, the extract significantly

(P<0.05) decreased IL-1α content by 50% at the highest extract concentration (0.5 mg/ml), while caspase-3 activity also remained unchanged (Table 4.4).

4.4.1.1 Effects of pre-exposure of the extracts on cell viability, IL-1α release and apoptosis following UVB-exposure

(i) Green tea

Pre-exposure of the keratinocytes to the green tea extract and the subsequent exposure to UVB light, decreased cell viability in a dose dependent manner (Table 4.5) when compared to the positive control. The IL-1-α content (pg/ml) and the normalised content decreased significantly (P<0.05) with increasing extract concentrations. Intracellular IL-1α was decreased to 1.5-fold at the highest concentration of the extract as compared to the 5.8 fold increase of the positive control. The extract significant decreased caspase-3 activity in a dose manner when compared to the UVB positive control, although the differences between the normalised data were not statistically significant.

(ii) Rooibos

Pre-exposure of HaCaT cells to varying concentrations of the rooibos extract displayed no significant effect on cell viability following UVB irradiation (Table 4.5). The IL-1α (pg/ml) as well as normalised IL-1α content was significantly (P<0.05) decreased at the two highest concentrations. Caspase-3 activity was significantly decreased (P<0.05) only at the highest concentration (0.2 mg/ml) of the rooibos extract, while the remaining concentrations displayed no significant effect when compared to the positive control.

(iii) Honeybush

Pre-exposure of HaCaT cells to *C. subternata* followed by UVB exposure, did not induce any significant effects on cell viability when compared to positive control. The pre-treatment with *C. subternata* resulted in a significant (P<0.05) decrease in IL-1α (pg/ml) as well as the normalised content at all concentrations used. Caspase-3 activity was also decreased significantly at the

Table 4.5 Effect of green tea and rooibos on cell viability, IL-1a accumulation and apoptosis of HaCaT cells exposed to UVB light

Biological endpoints	Controls		Green tea (mg/ml)		
	Negative Control	**Positive Control**	**0.05**	**0.1**	**0.2**
ATP content (% of control)	100.0 ± 4.6^{A}	$83.7\pm9.6^{A}_{a}$	$80.5\pm9.3_{a}$	$77.1\pm9.0_{a}$	$68.9\pm8.7_{b}$
IL-1α (pg/ml)	7.5 ± 0.9^{A}	$28.5\pm1.2^{B}_{a}$	$24.5\pm4.6_{a}$	$18.3\pm3.7_{a}$	$11.2\pm0.9_{b}$
Normalised IL-1α (pg/ml)*	7.5 ± 0.9^{A}	$42.0\pm10.7^{B}_{a}$	$32.5\pm1.9_{b}$	$26.3\pm1.3_{c}$	$19.0\pm3.9_{d}$
IL-1α (Fold decrease)	1.0 ± 0.1^{A}	$5.8\pm1.9^{B}_{a}$	$4.5\pm0.7_{a}$	$3.2\pm0.1_{a}$	$1.5\pm0.1_{b}$
Caspase-3 (Fold Increase)	1.0 ± 0.1^{A}	$6.2\pm0.5^{B}_{a}$	$5.0\pm0.9_{b}$	$4.3\pm0.7_{b}$	$3.4\pm1.1_{c}$
Normalised Caspase-3 (Fold Increase)**	1.0 ± 0.1^{A}	$7.4\pm0.9^{B}_{a}$	$6.1\pm0.5_{a}$	$5.6\pm1.2_{a}$	$5.0\pm2.3_{a}$
	Controls		**Rooibos (mg/ml)**		
	Negative Control	**Positive Control**	**0.025**	**0.05**	**0.1**	**0.2**
ATP content (% of control)	100.0 ± 4.6^{A}	$83.7\pm9.6^{A}_{a}$	$86.0\pm8.7_{a}$	$83.5\pm8.8_{a}$	$80.3\pm9.0_{a}$	$75.2\pm9.8_{b}$
IL-1α (pg/ml)	7.5 ± 0.9^{A}	$28.5\pm1.2^{B}_{a}$	$27.1\pm2.9_{a}$	$23.4\pm3.0_{a}$	$20.7\pm3.3_{b}$	$16.8\pm2.9_{b}$
Normalised IL-1α (pg/ml)*	7.5 ± 0.9^{A}	$42.0\pm10.7^{B}_{a}$	$36.4\pm5.9_{a}$	$32.4\pm3.1_{a}$	$29.1\pm2.3_{b}$	$25.0\pm2.5_{b}$
IL-1α (Fold decrease)	1.0 ± 0.1^{A}	$5.8\pm1.9^{B}_{a}$	$5.1\pm1.5_{a}$	$4.3\pm1.2_{a}$	$4.0\pm0.7_{a}$	$3.1\pm0.7_{a}$
Caspase-3 (Fold Increase)	1.0 ± 0.1^{A}	$6.2\pm0.5^{B}_{a}$	$5.6\pm1.1_{a}$	$5.5\pm1.4_{a}$	$6.0\pm0.6_{a}$	$4.0\pm0.4_{b}$
Normalised Caspase-3 (Fold Increase)**	1.0 ± 0.1^{A}	$7.4\pm0.9^{B}_{a}$	$5.7\pm1.1_{a}$	$6.5\pm1.5_{a}$	$7.0\pm0.7_{a}$	$4.0\pm0.8_{b}$

Cells were incubated at 37°C for 6 hrs. After the 6 hrs incubation, varying concentrations of green tea and rooibos extracts were added and the cells were then incubated for an additional 18 hrs; irradiated cultures were incubated for an additional 24 hrs. Values represent mean ± standard deviation of at least five replications; different uppercase letters in superscript in a row indicate differences between negative control and positive control; different lowercase letters in subscript in a row indicate differences between the positive control and concentrations of anti-inflammatory drugs. *Normalised IL-1α was calculated by correcting the IL-1α according to cell viability (section 4.2.3.2.2); **Normalised caspase fold increase is the caspase fold increase normalised by using the percentage ATP content (section 4.2.3.2.3). Significant differences was consider at $P<0.05$.

Table 4.6 Effect of *C. subternata* and *C. maculata* on cell viability, IL-1a accumulation and apoptosis of HaCaT cells exposed to UVB light

Biological endpoints	Controls		*Cyclopia subternata* (mg/ml)		
	Negative Control	**Positive Control**	**0.1**	**0.2**	**0.4**
ATP content (% of control)	100.0 ± 4.6^{A}	$83.7\pm9.6^{A}_{a}$	$82.8\pm8.1_{a}$	$82.5\pm6.5_{a}$	$81.1\pm6.0_{a}$
IL-1α (pg/ml)	7.5 ± 0.9^{A}	$28.5\pm1.2^{B}_{a}$	$17.6\pm1.6_{b}$	$14.7\pm1.2_{c}$	$12.5\pm0.8_{c}$
Normalised IL-1α (pg/ml)*	7.5 ± 0.9^{A}	$42.0\pm10.7^{B}_{a}$	$24.4\pm3.1_{b}$	$20.5\pm3.7_{b}$	$18.6\pm3.0_{b}$
IL-1α (Fold decrease)	1.0 ± 0.1^{A}	$5.8\pm1.9^{B}_{a}$	$3.0\pm0.9_{b}$	$2.5\pm0.9_{b}$	$2.1\pm0.6_{b}$
Caspase-3 (Fold Increase)	1.0 ± 0.1^{A}	$6.2\pm0.5^{B}_{a}$	$5.8\pm1.5_{a}$	$4.1\pm0.5_{b}$	$3.0\pm0.4_{b}$
Normalised Caspase-3 (Fold Increase)**	1.0 ± 0.1^{A}	$7.4\pm0.9^{B}_{a}$	$6.9\pm1.5_{a}$	$4.8\pm0.5_{a}$	$4.0\pm0.2_{b}$
	Controls		*Cyclopia maculata* (mg/ml)			
	Negative Control	**Positive Control**	**0.0625**	**0.125**	**0.25**	**0.5**
ATP content (% of control)	100.0 ± 4.6^{A}	$83.7\pm9.6^{A}_{a}$	$83.8\pm10.4_{a}$	$78.4\pm9.6_{a}$	$79.7\pm8.0_{a}$	$69.6\pm7.6_{b}$
IL-1α (pg/ml)	7.5 ± 0.9^{A}	$28.5\pm1.2^{B}_{a}$	$24.5\pm3.4_{a}$	$21.4\pm3.8_{b}$	$19.1\pm5.2_{b}$	$13.4\pm2.3_{c}$
Normalised IL-1α (pg/ml)*	7.5 ± 0.9^{A}	$42.0\pm10.7^{B}_{a}$	$34.5\pm5.4_{a}$	$30.9\pm2.2_{b}$	$27.7\pm0.9_{b}$	$22.0\pm3.9_{c}$
IL-1α (Fold decrease)	1.0 ± 0.1^{A}	$5.7\pm1.9^{B}_{a}$	$4.3\pm1.2_{a}$	$3.8\pm0.9_{a}$	$3.4\pm0.2_{a}$	$1.9\pm0.1_{b}$
Caspase-3 (Fold Increase)	1.0 ± 0.1^{A}	$6.2\pm0.5^{B}_{a}$	$4.8\pm0.3_{b}$	$4.9\pm1.3_{b}$	$3.9\pm0.8_{b}$	$3.5\pm1.0_{b}$
Normalised Caspase-3 (Fold Increase)**	1.0 ± 0.1^{A}	$7.4\pm0.9^{B}_{a}$	$5.1\pm0.4_{b}$	$6.1\pm1.4_{a}$	$4.5\pm0.6_{b}$	$3.4\pm0.2_{b}$

Cells were incubated at 37°C for 6 hrs. After the 6 hrs incubation, varying concentrations of *C. subternata* and *C. maculata* extracts were added and the cells were then incubated for an additional 18 hrs; irradiated cultures were incubated for an additional 24 hrs. Values represent mean ± standard deviation of at least five replications; different uppercase letters in superscript in a row indicate differences between negative control and positive control; different lowercase letters in subscript in a row indicate differences between the positive control and concentrations of anti-inflammatory drugs. *Normalised IL-1α was calculated by correcting the IL-1α according to cell viability (section 4.2.3.2.2); **Normalised caspase fold increase is the caspase fold increase normalised by using the percentage ATP content (section 4.2.3.2.3). Significant differences was consider at $P<0.05$.

two highest concentrations of the extracts, but when the data were normalised against cell viability, caspase-3 activity was only decreased significantly at 0.4 mg/ml (Table 4.6). In HaCaT cells exposed to *C. maculata* the viability of the keratinocytes significantly decreased at the highest concentration (Table 4.6). Intracellular IL-1α content (pg/ml) as well as the normalised IL-1α content was decreased ($P<0.05$) from 0.125 mg extract/ml and higher. The caspase-3 fold significantly decreased at all the extract concentrations and reaching significance ($P<0.05$) at the two highest concentrations when normalised according to cell viability.

4.4 Discussion

Exposure to UVB mainly penetrates into the epidermis and resulted in the generation of oxidative stress in keratinocytes, the predominant cells constituting this skin layer. UVB exposure results in numerous adverse effects including DNA damage and changes to the cellular redox status resulting in the generation of reactive oxygen species (ROS), the prolonged release of which will lead to additional tissue damage and chronic inflammation one of the hallmark of skin carcinogenesis (Wagener et al., 2013). Excessive ROS production and the depletion of glutathione levels result in the modification of lipid, protein and DNA structure and function. Defects in antioxidant defence mechanisms resulting in the deregulation of cell signalling pathways and the triggering downstream signalling cascades leading to altered cytokine release and exacerbation of inflammation are also adverse effects of high ROS levels in cells. The subsequent ROS mediated signal transduction stimulated pathways leads to the activation of activor protein-1(AP-1) and nuclear factor-kappaB (NF-κB) transcription factor complexes which regulates the release of important cytokines including IL-1α and TNF-α (Kramer-Stickland et al., 1999). Of interest is that the release of iron from ferritin stores also participate in the UVB related oxidative damage and the activation of the abovementioned transcription factors related to the induction of inflammatory responses (Wagener et al., 2013). The regulation of oxidation stress and the subsequent stimulation of inflammatory responses have been the target of many chemopreventive approaches, specifically focussing on the introduction of antioxidant and iron chelating agents in the

prevention of UV-induced inflammatory responses. Many food components, including tea polyphenols have been suggested to protect against UVB-induced adverse effects in the skin by altering the oxidative stress-mediated responses. (Aggarwal and Shishodia, 2006; Fresco et al., 2010; Mantena and Katiyar, 2006; Mukhtar and Ahmad, 2000; Potapovich et al., 2011).

A UVB keratinocyte model utilising human keratinocytes (HaCaT) was recently developed to investigate the anti-inflammatory effects of rooibos and honeybush herbal teas using green tea as benchmark (Magcwebeba et al., 2012; Magcwebeba, 2013). Extracts of these herbal teas were shown to remove UVB–exposed keratinocytes, containing high levels of the pro-inflammatory cytokine IL-1α by enhancing apoptosis in a post-exposure UVB model, implying an indirect anti-inflammatory mode of action. It was speculated that this was due to the pro-oxidant nature of the polyphenolic constituents enhancing apoptosis. The present study was designed to develop a cell culture system for monitoring the preventive action of anti-inflammatory compounds and plant extracts by establishing a pre-exposure model in the HaCaT keratinocytes. It was important to minimise direct interactions between polyphenols and UVB-induced oxidative stress effects which provide a masking effect when assessing the anti-inflammatory effect of the teas and herbal teas (Magcwebeba, 2013). In order to modify the post-exposure model, it was important to determine an optimal method to deliver the extracts to the cells without influencing cell viability and elicit a similar immune response after exposure UVB as was recorded in the post-exposure model. Thus, it was prudent for the cells to adhere to the wells as well as to be treated with the extracts without adversely affecting the cells. The 6 hrs seeding period followed by an 18 hrs pre-treatment period with the tea and herbal tea extracts provided suitable conditions to monitor possible anti-inflammatory effects of the tea and herbal tea extracts in the newly developed pre-exposure HaCaT model.

In order to validate the pre-exposure model as a means to investigate the preventive anti-inflammatory properties of any compound or solution, known anti-inflammatory compounds, dexamethasone and ibuprofen were screened for their anti-inflammatory abilities utilising the newly developed cell culture model. The cells

were treated with the compounds using concentrations shown previously to inhibit the production of IL-1α utilising the post-exposure model (Magcwebeba, 2013). However, the anti-inflammatory agents did not have the expected effect of reducing the accumulation of intracellular IL-1α. Both dexamethasone and ibuprofen are generally known as therapeutic anti-inflammatory compounds and it would appear that, when added to cells before an inflammatory stimulus, they lack the reduction of UVB-induced IL-1α accumulation and were thus not able to reduce inflammation. Despite the lack of the anti-inflammatory effect of these drugs it was of interest to screen the tea and herbal extracts for their potential to prevent inflammation in the developed pre-exposure model.

Green tea has been shown to modulate UVB-induced inflammation by inhibiting leukocyte infiltration and ROS production in mouse skin (Katiyar et al., 1999b). Topical application of green tea to mouse skin has been shown to inhibit UVB-induced immunosupression by inducing the immunoregulatory cytokine, IL-12 (Katiyar, 2011). Both the abovementioned abilities of green tea were attributed to the presence of EGCG, the most prevalent catechin present. In this study pre-exposure of the cells to green tea extract reduced UVB-induced IL-1α accumulation, while maintaining cell viability comparable to that of the positive UVB-treated control. The extract also had an inhibitory effect on caspase-3 activity, which indicates its ability to prevent the cells from undergoing apoptosis, a common outcome following UVB exposure (Tomas, 2009). The 40% reduction of IL-1α prior to the UVB exposure, in the absence of reduced cell viability and apoptosis leads to the notion that the green tea extract exhibited a reduction in baseline IL-1α content implying an anti-inflammatory effect. A possible “sunscreen” effect, following the removal of the extract containing culture media, may reduce the effect of the UVB dose towards the pre-exposed HaCaT cells still containing residual polyphenolic constituents. However, the reduction of IL-1α by green tea after the 18 hrs pre-exposure prior to the UVB treatment seems to indicate a direct anti-inflammatory effect on baseline cellular IL-1α content. This is further emphasised by evidence suggesting that the green extract, presumably EGCG, protects against the adverse effects of UV irradiation via the inhibition of the translocation of NF-κB in normal keratinocytes (Afaq et al., 2003).

Rooibos exhibits a plethora of biological properties, including antioxidant, anti-inflammatory, immunomodulatory, anti-proliferative and anticancer activities (Joubert et al., 2008). The anticancer properties of rooibos have been demonstrated in mouse skin indicating that a rooibos extract exhibited chemoprotective effects (Marnewick et al., 2005, 2009). Rooibos also significantly reduced IL-1α induction in HaCaT cells treated for 18 hrs prior to UVB exposure demonstrating anti-inflammatory properties. As described for green tea, the rooibos extract did not alter cell viability, except at the highest concentration used, and which was associated with an increase in apoptosis. Of the rooibos flavonoids, luteolin, quercetin and its rutinoside, rutin, have been reported to reduce the expression of NF-κB in keratinocytes without affecting ATP production and cell viability (Potapovich et al., 2011; Vicentini et al., 2011; Weng et al., 2014). It is therefore possible that the rooibos extract elicited an anti-inflammatory effect, however, a sunscreen effect which may significantly reduce the effective UV dose, cannot be ruled out and requires further investigations.

Honeybush also possess antioxidant, anti-inflammatory, immunomodulatory, anti-proliferative and anticarcinogenic activities (Joubert et al., 2008). Extracts of honeybush and their major polyphenolic constituents, mangiferin and hesperidin demonstrated the modulation of skin carcinogenesis in mice and photoprotective effects in mouse skin (Petrova et al., 2011). The *C. subternata* extract used in the current study did not display any effect on cell viability and apoptosis whilst baseline or constitutive IL-1α levels were significantly reduced prior to UVB exposure. The extract also reduced IL-1α accumulation and caspase-3 activity following UVB exposure, whilst cell viability remained unchanged. As the extract did exhibit a 2-fold reduction in IL-1α accumulation a “memory” effect seems to prevail following the pre-exposure of the HaCaT cells, whereby the cells that are exposed to UVB contain significantly less IL-1α during irradiation. Of the honeybush polyphenols mangiferin has been shown to exhibits anti-inflammatory properties (Gong et al., 2013).

Several mechanism have been proposed whereby polyphenols inhibited the UVB-induced cytokine induction in keratinocytes which are mainly related to the induction of oxidative stress and the activation of AP-1 and NF-κB pathways through signal

transduction pathways. The antioxidant properties of the tea and herbal tea extracts therefore, have been implicated to play a determining role when considering the pre-exposure UVB model.

It is known that polyphenols protect against the increased depletion of vitamin E and C in membranes due to their low redox potential (Wagener et al., 2013). The polyphenols also has been implicated in their role as pro-oxidants, which, depending on the concentration, could further exaggerated the UVB-induced oxidative stress as was hypothesised in the post-exposure UVB model (Magcwebeba, 2013). However, in the current study pre-exposure and the subsequent removal of the culture medium containing the extract appears to suggest that far lower levels of the polyphenols are available and that their antioxidant properties tend to prevail. Countering the UVB-induced oxidative response therefore could be an important mechanism to minimise the over production of oxygen radicals likely to reduce the stimulation of NFκB and the subsequent induction of IL-1α build up within the keratinocytes. A second mechanism that could be involved is related to the iron release from ferritin associated with the increased oxidative stress in keratinocytes (Pelle et al., 2011). Intracellular iron release may contribute to ROS production induced by UVB exposure via the Fention reaction and thus increase inflammatory response (Kramer-Stickland et al., 1999). These interactions, discussed in detail in Chapter 3, should therefore become important tools in the inhibition of UVB-induced inflammation in the skin. In this regard mangiferin has been implicated to play an important role in the stabilisation of free iron preventing it to be involved in subsequent free radical reactions (Matkowski et al., 2013).

The pre-exposure model developed in this study provided opportunities to further investigate the mechanisms in reducing the UVB-induced IL-1α accumulation via the anti-inflammatory effect by the tea and herbal teas. However, it also presents challenges such as potential sunscreen effects that might play a role in the protection against the UVB-induced effect on the accumulation of IL-1α. Future studies on the preventive anti-inflammatory effects of these extracts should include the role of the individual herbal tea polyphenols in the protection against UVB-

induced inflammation. Flow cytometry can also be employed to verify the state of the cells, whether they are in active apoptosis, necrosis or still viable. This will enable a more conclusive deduction regarding the protective and/or anti-inflammatory effects of the herbal extracts in keratinocytes.

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Chapter 5

THE INHIBITORY POTENTIAL OF GREEN TEA AND HERBAL TEA EXTRACTS AGAINST LIPOPOLYSACCHARIDE-INDUCED INFLAMMATION IN THP-1 DERIVED MACROPHAGES

ABSTRACT

Tumour necrosis factor alpha (TNF-α), a primary cytokine released by macrophages, can mediate cancer when not regulated and secreted into circulation. Multiple intracellular signals are induced by TNF-α, including signals for cell survival through nuclear factor kappa B (NF-κB) and cell death through caspase activation. During chronic inflammation, the imbalance between anti-apoptotic and pro-apoptotic signalling by TNF-α may lead to the dysregulation of cell survival and proliferation and tumour formation. Polyphenols, present in fruits, vegetables and beverages from plants, have shown efficacy against chronic diseases mainly due to their ability to alter pro-inflammatory pathways via the modulation of both the production and action of inflammatory molecules. This study focuses on the ability of green tea (*Camellia sinensis*) and unfermented rooibos (*Aspalathus linearis*) and honeybush (*Cyclopia spp.*) aqueous extracts to reduce TNF-α release by monocyte (THP-1) derived macrophages exposed to lipopolysaccharide (LPS). *In vitro* models were developed to investigate the effect of the extracts on the release of TNF-α, cell viability and apoptosis when the macrophages are treated with the extracts prior to (pre-exposure) and simultaneous to (co-exposure) immune response stimulation. Findings of the current study suggest that pre-exposure provide a better approach to explore the anti-inflammatory properties of the herbal tea extracts as co-exposure could result in increased oxidative stress parameters leading to apoptosis. The importance of NFκB expression and translocation in mediating the inflammation process still has to be investigated to further elucidate the mechanism of the ability of the extracts to reduce TNF-α.

5.1. Introduction

Inflammation is the body's first line of defence against injury, cell damage, ultra violet light B (UVB) exposure and infection (Mak and Saunders, 2006). Macrophages play an important role in the process of inflammation and reside in all organs and tissues, usually in the locations closest to where inflammation might occur (Cavaillon, 1994). They are phagocytes, producing growth factors that stimulate cells involved in wound healing (Mak and Saunders, 2006). Upon stimulation they secrete a host of cytokines, including interleukin-1 (IL-1) and tumour necrosis factor α (TNF-α) that attract other immune cells to the site of inflammation (Cavaillon, 1994). TNF-α, induced by a wide range of pathogenic stimuli, induces other inflammatory mediators and proteases that orchestrate inflammatory responses (Feghali and Wright, 1997).

Acute inflammation, a normally rapid process, may become chronic if it is maintained for a prolonged period (Mueller, 2006). Chronic inflammation leads to the development of many diseases which include cancer (Aggarwal et al., 2006). During chronic inflammation the dysregulation of TNF-α has been linked to all steps involved in tumourigenesis and is known to be present in multiple tumours (Mantovani et al., 2008). Multiple intracellular signals are induced by TNF-α, including signals for cell proliferation through nuclear factor kappa B (NF-κB) and cell death through caspase activation (Wang et al., 1996). During chronic inflammation, a shift in this balance occurs and leads to the development of tumours (Cooper and Caligiuri, 2003).

Plants produce polyphenols as secondary metabolites (Pandey and Rizvi, 2009) and consumed in the human diet via edible plants such as fruit, vegetables and various herbal infusions (Fraga et al., 2010; Lambert and Elias, 2010; Rice-Evans et al., 1997, 1996). These compounds are known to be effective treatments against chronic diseases mainly due to their anti-inflammatory properties (Gupta et al., 2014). Green tea (*Camellia sinensis*) and its catechins also have the ability to modulate the immune responses (Cabrera et al., 2006). Rooibos (*Aspalathus linearis*) extracts stimulate the production of immunoglobulin M (IgM) associated with IL-10 production (Ichiyama et al., 2007). Mangiferin, a major honeybush (*Cyclopia* spp.) xanthone, reduces the production of cytokines such as TNF-α and

IL-6 (Gong et al., 2013) while other xanthones inhibit LPS-induced inflammation in human macrophages (Bumrungpert et al., 2010).

As chronic inflammation is known to be involved in cancer development (Mantovani et al., 2008; Medzhitov, 2010; Mueller, 2006; Multhoff et al., 2011) it has become a potential target in the prevention and eradication of cancer. Since macrophages and TNF-α, play such an integral part in acute and chronic inflammation, this chapter focuses on the ability of green tea and rooibos and honeybush extracts to reduce TNF-α release by monocyte (THP-1) derived macrophages. The modulating role of the different plant extracts was critically assessed in relation to the effect on cell viability, the inhibition of TNF-α release and the induction of apoptosis. The development of *in vitro* cell culture models and the screening of the extracts utilizing a pre and co-exposure approach will be presented.

5.2. Materials and Methods

5.2.1. Chemicals

Bovine serum albumin (BSA), dimethyl sulfoxide (DMSO), Dulbecco's phosphate buffered saline (DPBS), dexamethasone, ibuprofen, phorbol 12-myristate 13-acetate (PMA) and lipopolysaccharides (LPS) were purchased from Sigma-Aldrich (St. Louis, USA). Heat inactivated fetal bovine serum (FBS) was obtained from HyClone (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA). RPMI-1640, ultra-glutamine, trypsin-versene and Hank's buffered salt solution (HBSS) were obtained from Lonza (Basel, Switzerland). Human recombinant tumour necrosis factor-α (TNF-α) ELISA kit was purchased from R&D systems (Minneapolis, USA) and CellTiter-Glo luminescent cell viability and Caspase-3/7 assays were purchased from Promega (Madison, USA). TritonX-100 was obtained from Roche (Basel, Switzerland).

5.2.2. Plant material and preparation of extracts

The different plant materials and their aqueous extracts were described in detail in Chapter 3.

5.2.3. THP-1 cell maintenance, experimental procedures and biological endpoints measured

THP-1 human monocytic leukemia cells were obtained from the European Collection of Cell Cultures (ECACC, Salisbury, UK) and maintained in RPMI-1640 supplemented with 10% FBS (v/v) and 2 mM ultra-glutamine in a humidified atmosphere of 5% CO_2/95% air at 37°C. Cells were maintained as suspension cultures at an optimal density ranging from 3 to 9 x 10^5 cells/ml as prescribed by the supplier.

5.2.3.1. Determination of cell viability (ATP content)

Cell viability was determined, in terms of the ATP content as described in Chapter 4 (Section 4.2.3.2.1), using the CellTiterGlo kit according to the manufacturer's instructions.

5.2.3.2. Determination of TNF-α release

TNF-α, which is released into the supernatant by the cells, was determined utilising a TNF-α ELISA kit (R&D systems, Minneapolis, USA) according to the procedures defined by the manufacturer. A standard curve was generated using human recombinant TNF-α prepared in 1% BSA in DPBS (w/v) using seven concentrations ranging from 15.6 to 1000 pg/ml. Absorbance was measured at 450nm with a Synergy 2 Multi-Mode Microplate Reader (BioTek®, Winooski, Vermont, USA) and data were analysed using the standard curve generated from Gen5™ Data Analysis Software (version 2 for Windows). TNF-α was expressed as

pg/ml, normalised against ATP content as a surrogate measure for cell viability and also expressed as a percentage of the positive control. The following equations were utilised:

$$\text{Normalised TNF-}\alpha = \frac{\text{TNF-}\alpha\text{ (sample) in pg/ml}}{\text{Cell viability in \% of control}} \times 100$$

$$\%\ \text{TNF-}\alpha\text{ (of positive control)} = \frac{\text{TNF-}\alpha\text{ (sample)}}{\text{TNF-}\alpha\text{ (positive control)}} \times 100$$

5.2.3.3. Determination of apoptosis

Apoptosis in the cell lysates was determined as described in Chapter 4 (Section 4.2.3.2.3), Analyses were conducted using a Veritas™ microplate luminometer (Turner Biosystems) and the data expressed as fold increase of the positive control, representing the augmentation of apoptosis in cells. The fold increase was also normalised against ATP content as a surrogate measure for cell viability. The following equations were utilised:

$$\text{Caspase Fold Increase} = \frac{\text{RLU (sample)}}{\text{RLU (positive control)}}$$

$$\text{Normalised Caspase Fold Increase} = \frac{\text{Caspase Fold Increase}}{\text{Cell viability in \% of control}} \times 100$$

5.2.3.4. Development of macrophage inflammatory model utilising THP-1 monocytes for the evaluation of anti-inflammatory properties of tea and herbal tea extracts

(i) Determination of optimal conditions of monocyte differentiation by DMSO and PMA

THP-1 monocytes were seeded at a density of 2 x 10^4 cells/well in a 96-well microtiter plate (Corning Costar, New York, USA) using RPMI-1640 media containing 10% FBS (v/v) and either 1% DMSO (v/v), 2.5% DMSO (v/v), 5% DMSO (v/v), 100 ng/ml PMA or 200 ng/ml PMA and incubated for 72 hrs at 37°C. The cells were washed using HBSS and the medium replaced with RPMI-1640 media containing 0.5% FBS (v/v) and incubated for an additional 24 hrs resting period, after which cell viability was determined.

(ii) Determination of optimal conditions for LPS stimulation of TNF-α excretion

Following differentiation, the macrophages (2 x 10^4 cells/well) were washed with HBSS and incubated with RPMI-1640 media (100 µl) containing 0.5% FBS (v/v). For TNF-α induction, a range of LPS concentrations (5, 10 or 20 ng/ml) were prepared in the culture medium containing 0.5% FBS (v/v) and the cells incubated for 2, 4, 6 or 24 hrs using the culture medium as a control and TNF-α release determined. Cell viability was determined separately.

5.2.3.5. Model validation utilising anti-inflammatory drugs

(i) Pre-exposure model

Macrophages were firstly exposed to the anti-inflammatory compounds after which inflammation was induced by LPS. Macrophages were incubated with RPMI-1640 media containing 0.5% FBS (v/v) and 0.5% DMSO (v/v) and varying concentrations of dexamethasone or ibuprofen (5 concentrations between 1 pM and 1 nM) for 24 hrs. The media containing the anti-inflammatory compounds were removed and RPMI-1640 medium containing 0.5% FBS and 10 ng/ml LPS added and incubated for 6 hrs. Macrophages exposed to medium containing 0.5% DMSO

and 0.5% FBS served as negative control. Macrophages exposed to LPS in the absence of the anti-inflammatory compounds was used as a positive control. The supernatants were then transferred to 96-well plates and TNF-α was determined as described above. Cell viability was determined separately.

(ii) Co-exposure model

Macrophages were exposed to the anti-inflammatory compounds and LPS simultaneously. Following macrophage differentiation after the 24 hrs resting period, RPMI-1640 medium containing 0.5% FBS (v/v), 10 ng/ml LPS and dexamethasone or ibuprofen (five concentrations ranging from 0.001 to 1nM) were added and macrophages incubated for 6 hrs after which TNF-α release and cell viability were determined. Macrophages incubated with the culture medium and LPS, in the absence of the anti-inflammatory compounds, served as negative and positive controls, respectively.

5.2.4. Determination of the effect of tea and herbal tea extracts on cell survival and inflammation induced by LPS in THP-1 derived macrophages

A schematic diagram of the inflammation model developed and the methodology to screen for the anti-inflammatory effects of green tea, rooibos and the two *Cyclopia* herbal teas are outlined below.

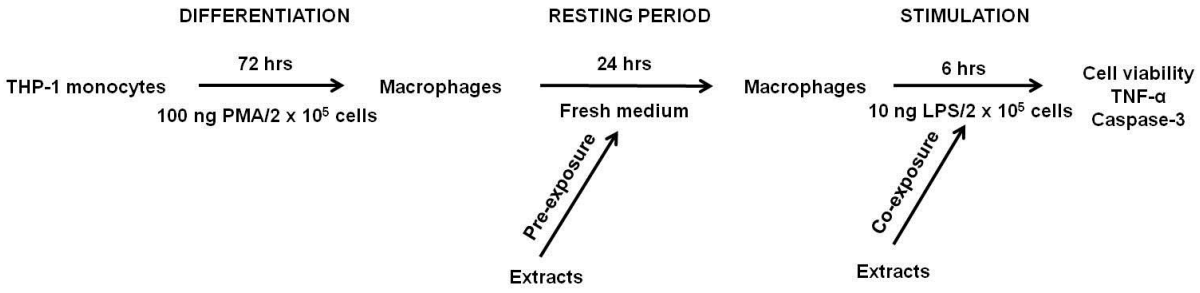

Fig 5.1 Schematic outline of the inflammatory macrophage TNF-α model monitoring the anti-inflammatory effects of the tea and herbal tea extracts utilising pre- and co-exposure modes of exposure.

5.2.4.1. Preparation of extracts.

Freeze-dried extracts of green tea, rooibos and the two honeybush spp. were dissolved in DMSO and diluted in RPMI-1640 medium containing 0.5% FBS (v/v) to a concentration of 40 mg extract/ml (10% DMSO (v/v)). The extracts were diluted again with RPMI-1640 to a concentration of 2 mg extract/ml containing a final DMSO concentration of 0.5% (v/v) and filtered (0.22 µm). The filtered solutions and RPMI-1640 medium containing 0.5% FBS (v/v) and 0.5% DMSO (v/v) were used to prepare the different concentrations of each extract to be tested in the macrophage model. These concentrations were determined based on the IC_{50} values of the extracts inhibiting 50% of the cell viability of the macrophages (see below).

5.2.4.2. Determination of the effect of green tea and herbal extracts in a pre-exposure macrophage model

(i) Determination of the effect of the extracts on cell survival indices in macrophages before LPS stimulation

After monocyte differentiation, the medium was removed, replaced with RPMI-1640 medium containing 0.5% FBS and varying concentrations of the extracts, after which the cells incubated for 24 hrs. The cells were lysed with a combination of 0.5% TritonX-100 (v/v) in DPBS, pH 7.4 and stored at -80°C until analysis. Cell viability was determined separately and caspase-3 activity was determined in the cell lysates as described above. Macrophages incubated with the culture medium, in the absence of the extracts, served as control. The concentration of each extract required to reduce cell viability by 50% (IC_{50}) were calculated using GraphPad Software Prism Version 5.00 for Windows (GraphPad Software, La Jolla, California, USA).

(ii) Determination of the modulation of TNF-α release in macrophages following pre-exposure to the extracts

Macrophages were incubated with RPMI-1640 medium containing 0.5% FBS (v/v), 0.5% DMSO (v/v) and the different dilutions of the extracts during the resting period for 24 hrs. The medium was subsequently replaced with RPMI-1640 medium containing 0.5% FBS (v/v) and 10 ng/ml LPS and incubated for 6 hrs. Macrophages exposed to neither extracts nor LPS was used as a negative control. Macrophages exposed to LPS in the absence of the extracts, was used as the positive control. The supernatants were then transferred to 96-well plates and TNF-α was determined as described above. The cells were lysed with a combination of 0.5% TritonX-100 (v/v) in DPBS, pH 7.4 and stored at -80°C until analysis. TNF-α release was determined in the supernatant and caspase-3 activity was determined in the cell lysates as described above. Cell viability was determined separately.

(iii) Determination of the effect of green tea and herbal tea extracts in the co-exposure macrophage model

After removing the medium from differentiated macrophages, cells were incubated with RPMI-1640 medium containing 0.5% FBS (v/v), 0.5% DMSO (v/v), 10 ng/ml LPS and the extracts for 6 hrs. Macrophages cultured in the absence of the extracts and LPS served as negative controls, whereas macrophages exposed to LPS in the absence of the extracts were used as a positive control. The supernatants were transferred to 96-well plates for the determination of TNF-α release as described above. The cells were lysed with a combination of 0.5% TritonX-100 (v/v) in DPBS, pH 7.4 and stored at -80°C until analysis. TNF-α release was determined in the supernatant and caspase-3 activity was determined in the cell lysates as described above. Cell viability was determined separately.

5.2.5. Statistical analysis

Descriptive statistics were performed on the data as described in Chapter 4 (Section 4.2.5)

5.3. Results

5.3.1. Establishment and evaluation of the inflammatory model

5.3.1.1. Determination of optimal conditions for monocyte differentiation by DMSO and PMA

Although incubating monocytes in 1% DMSO did not affect the cell viability, it also did not result in any morphological changes associated with monocyte differentiation. The cells remained round and did not adopt the typical morphology of macrophages (data not shown). The treatment of 2.5% and 5% DMSO resulted in a significant ($P<0.05$) decrease in cell viability, indicating that DMSO would be unsuitable for monocyte differentiation and model development. Incubating the monocytes in 100 ng/ml PMA for 72 hrs was the only treatment to result in the desired morphological changes and an increase in adherence of the cells to the culture dish without

effecting cell viability (Table 5.1). Monocyte differentiation with 200 ng/ml PMA also resulted in morphological changes, but displayed increased loss in cell viability, showing a concentration of 100 ng PMA/2 x 10^5 cells to be an adequate concentration for monocyte differentiation.

Table 5.2 Analyses of monocyte differentiation and cell viability

Compound	% ATP Content
Control	100.0 ± 7.9^{A}
DMSO (%)	
1	94.2 ± 4.6^{A}
2.5	19.8 ± 4.7^{B}
5	6.7 ± 1.6^{C}
PMA (ng/ml)	
100	144.5 ± 16.6^{D}
200	34.11 ± 6.6^{E}

Monocytes were incubated with the compound at the specified concentrations for 72 hrs. Values represent mean ± standard deviation of at least five replications. Different uppercase letters in superscript indicate significant (P<0.05) differences between untreated cells (control) and treated cells.

5.3.1.2. Analyses of LPS stimulation

Cell viability was significantly (P<0.05) reduced when cells were treated at the highest LPS concentration after 24 hrs only, while no significant changes were noticed at the other concentrations and incubation times (Table 5.2). TNF-α secretion between 4 hrs and 24 hrs of incubation were similar at 5 and 10 ng/ml LPS concentrations. However, stimulation with 10 ng/ml LPS did exhibit a higher degree of stimulation of TNF-α release, at both 4 and 6 hrs whilst maintaining comparable cell viability. A significant (P<0.05) decrease in TNF-α release was obtained at 20 ng/ml at 6 hrs, possibly due to experimental error or due to cellular effects resulting from cell exposure to high LPS concentration. The data nevertheless indicate that stimulation by LPS at a concentration of 10 ng/ml for 6 hrs is optimal for maximum TNF-α release.

Table 5.3 Analyses of LPS concentration and stimulation time on cell viability and TNF-α release

Stimulation time (hrs)	LPS (ng/ml)			
	Control	5	10	20
% ATP Content				
2	$100\pm5.7^{A}_{a}$	$97.6\pm8.0^{A}_{a}$	$96.9\pm8.1^{A}_{a}$	$103.3\pm8.7^{A}_{a}$
4	$100\pm6.8^{A}_{a}$	$86.0\pm8.7^{B}_{a}$	$88.7\pm8.7^{B}_{a}$	$88.0\pm9.0^{B}_{a}$
6	$100\pm4.4^{A}_{a}$	$87.1\pm7.8^{B}_{a}$	$81.4\pm9.8^{B}_{a}$	$81.6\pm9.8^{B}_{a}$
24	$100\pm5.4^{A}_{a}$	$87.6\pm9.8^{B}_{a}$	$87.5\pm5.7^{B}_{a}$	$74.9\pm12.5^{C}_{b}$
TNF-α release (pg/ml)				
2	$13.3\pm1.6^{A}_{a}$	$469.4\pm31.0^{B}_{a}$	$584.5\pm46.7^{B}_{a}$	$428.6\pm13.8^{B}_{a}$
4	$13.3\pm1.8^{A}_{a}$	$1221.6\pm180.0^{B}_{b}$	$1321.9\pm165.6^{B}_{b}$	$1206.7\pm175.8^{B}_{b}$
6	$13.4\pm1.4^{A}_{a}$	$1283.7\pm204.9^{B}_{b}$	$1440.1\pm111.2^{B}_{b}$	$772.3\pm145.1^{C}_{c}$
24	$13.5\pm1.7^{A}_{a}$	$988.5\pm288.2^{B}_{b}$	$1330.2\pm84.9^{C}_{b}$	$1278.5\pm146.8^{C}_{b}$

Macrophages incubated for 24 hrs in fresh medium and thereafter incubated with LPS at the specified concentrations for the specified periods. Values represent mean ± standard deviation of at least five replicates. Different uppercase letters in superscript (in a row) indicate differences between values at the same stimulation time ($P<0.05$); Different lowercase letters in subscript (in a column) indicate differences between values at the same LPS concentrations ($P<0.05$). The control group was incubated in fresh medium instead of LPS for the specified periods.

5.3.2. Validation of inflammation model utilising anti-inflammatory drugs

5.3.2.1. Analyses of cell viability and TNF-α in the pre-exposure model

The cell viability was not significantly affected by the different concentrations of the anti-inflammatory compounds, except for dexamethasone which decreased cell viability by approximately 50% at 1 nM. TNF-α release was significantly ($P<0.05$) decreased at the two highest concentrations (Table 5.3). Both anti-inflammatory compounds inhibited the direct and normalised (corrected according to cell viability) TNF-α levels up to 20% when cells were exposed to higher concentrations. The lower concentrations of both anti-inflammatory compounds did exhibited an inhibitory effect on TNF-α release.

5.3.2.2. Analyses of cell viability and TNF-α release in the co-exposure model

Analyses of the parameters showed that the anti-inflammatory compounds exhibited a dose-dependent inhibitory effect on TNF-α secretion under co-exposure conditions with LPS, considering the direct, normalised and % inhibition of TNF-α release (Table 5.4). Cell viability was not affected by either ibuprofen or dexamethasone, whilst ibuprofen tended to be more effective in its ability to decrease TNF-α release. Both compounds were more effective in the co-exposure model compared to the pre-exposure model.

Table 5.4 Analyses of anti-inflammatory compounds on cell viability and TNF- α release of macrophages in pre-exposure model

Biological Endpoints	Negative Control	Positive Control	Ibuprofen (nM)				
			0.001	0.25	0.5	0.75	1.0
% ATP content	102.1 ± 13.2^{A}	$100.0\pm4.4^{A}_{a}$	$97.9\pm14.3_{a}$	$96.7\pm7.8_{a}$	$98.4\pm9.7_{a}$	$93.5\pm7.4_{a}$	$91.0\pm12.1_{a}$
TNF-α (pg/ml)	24.9 ± 3.3^{A}	$544.5\pm24.9^{B}_{a}$	$488.4\pm40.8_{a}$	$470.4\pm29.9_{a}$	$430.5\pm32.7_{a}$	$390.6\pm19.8_{b}$	$364.4\pm29.1_{b}$
Normalised TNF-α* (pg/ml)	24.2 ± 1.8^{A}	$544.5\pm24.9^{B}_{a}$	$492.9\pm70.2_{a}$	$487.1\pm39.2_{a}$	$438.3\pm34.5_{a}$	$418.0\pm21.5_{a,b}$	$400.7\pm31.6_{b}$
% TNF-α**	4.5 ± 0.4^{A}	$100.0\pm3.5^{B}_{a}$	$90.4\pm10.8_{a}$	$88.9\pm5.5_{a}$	$79.8\pm5.9_{a}$	$76.4\pm3.9_{a,b}$	$74.4\pm5.1_{b}$
Biological Endpoints	**Negative Control**	**Positive Control**	**Dexamethasone (nM)**				
			0.001	**0.25**	**0.5**	**0.75**	**1.0**
% ATP content	102.1 ± 13.2^{A}	$100.0\pm4.4^{A}_{a}$	$97.6\pm11.0_{a}$	$97.1\pm11.1_{a}$	$101.0\pm13.2_{a}$	$79.2\pm12.0_{a}$	$47.7\pm6.9_{b}$
TNF-α (pg/ml)	24.9 ± 3.3^{A}	$544.5\pm24.9^{B}_{a}$	$502.4\pm30.8_{a}$	$476.0\pm42.4_{a}$	$437.7\pm44.3_{a}$	$425.3\pm70.2_{a}$	$203.5\pm25.0_{b}$
Normalised TNF-α* (pg/ml)	24.2 ± 1.8^{A}	$544.5\pm24.9^{B}_{a}$	$491.5\pm29.3_{a}$	$476.0\pm67.3_{a}$	$454.6\pm82.7_{a}$	$424.8\pm44.9_{a,b}$	$426.8\pm52.4_{b}$
% TNF-α**	4.5 ± 0.4^{A}	$100.0\pm3.5^{B}_{a}$	$91.1\pm6.0_{a}$	$87.8\pm10.6_{a}$	$83.1\pm3.1_{a}$	$79.1\pm9.0_{a,b}$	$75.9\pm9.3_{b}$

Macrophages were incubated for 24 hrs in fresh medium containing the anti-inflammatory compounds and incubated with 10ng/ml LPS for 6 hrs thereafter. Values represent mean ± standard deviation of at least five replications; Different uppercase letters in superscript in a row indicate differences between negative control (no LPS, no anti-inflammatory compounds) and positive control (LPS, no anti-inflammatory compounds); Different lowercase letters in subscript in a row indicate differences between the different concentrations of anti-inflammatory compounds ($P<0.05$). *Normalised TNF-α was calculated by correcting the TNF-α according to cell viability, ** % TNF-α calculated as a percentage of positive control TNF-α release.

Table 5.5 Analyses of anti-inflammatory compounds on cell viability and TNF release of macrophages in co-exposure model

Biological Endpoints	Negative Control	Positive Control	Ibuprofen (nM)				
			0.001	0.25	0.5	0.75	1.0
% ATP	108.7 ± 10.1^{A}	$100.0\pm7.1^{A}_{a}$	$102.9\pm10.9_{a}$	$102.7\pm10.9_{a}$	$96.4\pm8.9_{a}$	$97.5\pm11.1_{a}$	$89.9\pm11.5_{a}$
TNF-α (pg/ml)	76.1 ± 9.7^{A}	$740.\pm31.5^{B}_{a}$	$601.9\pm88.6_{a}$	$357.0\pm32.2_{b}$	$229.4\pm31.5_{c}$	$147.9\pm15.1_{d}$	$80.0\pm17.1_{e}$
Normalised TNF-α* (pg/ml)	65.7 ± 8.4^{A}	$740.0\pm31.5^{B}_{a}$	$536.0\pm78.9_{a}$	$319.0\pm28.7_{b}$	$229.8\pm31.5_{c}$	$146.9\pm15.0_{d}$	$88.7\pm19.0_{e}$
% TNF**	8.9 ± 1.1^{A}	$100.0\pm4.3^{B}_{a}$	$72.4\pm10.7_{a}$	$43.1\pm3.9_{b}$	$31.1\pm4.3_{c}$	$19.9\pm2.0_{d}$	$12.0\pm2.6_{e}$
Biological Endpoints	**Negative Control**	**Positive Control**	**Dexamethasone (nM)**				
			0.001	**0.25**	**0.5**	**0.75**	**1.0**
% ATP	108.7 ± 10.1^{A}	$100.0\pm7.1^{A}_{a}$	$101.5\pm13.3_{a}$	$98.3\pm9.4_{a}$	$97.9\pm9.6_{a}$	$96.1\pm7.9_{a}$	$96.5\pm7.9_{a}$
TNF-α (pg/ml)	76.1 ± 9.7^{A}	$740.\pm31.5^{B}_{a}$	$757.2\pm20.9_{a}$	$399.0\pm65.9_{b}$	$230.1\pm37.6_{c}$	$170.8\pm44.8_{d}$	$137.0\pm31.7_{e}$
Normalised TNF-α* (pg/ml)	65.7 ± 8.4^{A}	$740.0\pm31.5^{B}_{a}$	$725.9\pm20.1_{a}$	$395.6\pm65.3_{b}$	$233.4\pm38.1_{c}$	$171.8\pm45.0_{d}$	$149.3\pm34.5_{e}$
% TNF**	8.9 ± 1.1^{A}	$100.0\pm4.3^{B}_{a}$	$98.1\pm2.7_{a}$	$53.5\pm8.8_{b}$	$31.5\pm5.1_{c}$	$23.3\pm6.1_{c,d}$	$20.2\pm4.7_{d}$

Rested macrophages were incubated with 10 ng/ml LPS and the anti-inflammatory compounds for 6 hrs. Values represent mean ± standard deviation of at least five replications; Different uppercase letters in superscript in a row indicate differences between negative control (no LPS, no anti-inflammatory compounds) and positive control (LPS, no anti-inflammatory compounds); Different lowercase letters in subscript in a row indicate differences between the different concentrations of anti-inflammatory compounds ($P<0.05$). *Normalised TNF-α was calculated by correcting the TNF-α according to cell viability, ** % TNF-α calculated as a percentage of positive control TNF-α release.

5.3.3. *Analyses of the anti-inflammatory potential of green and herbal tea extracts in THP-1 derived macrophages utilising pre- and co- exposure models*

5.3.3.1. *Modulation of cell viability and apoptosis without LPS-induced TNF-α stimulation by green tea and herbal tea extracts (basal levels)*

(i) Green tea

In macrophages, green tea significantly decreased ($P<0.05$) the cell viability after 24 hrs of exposure at concentrations of 0.2 mg extract/ml and above (Fig. 5.2A). The green tea extract exhibited no significant effect on caspase-3 activity although it tended to stimulate apoptosis (not significantly) at the highest concentration (Fig 5.2B).

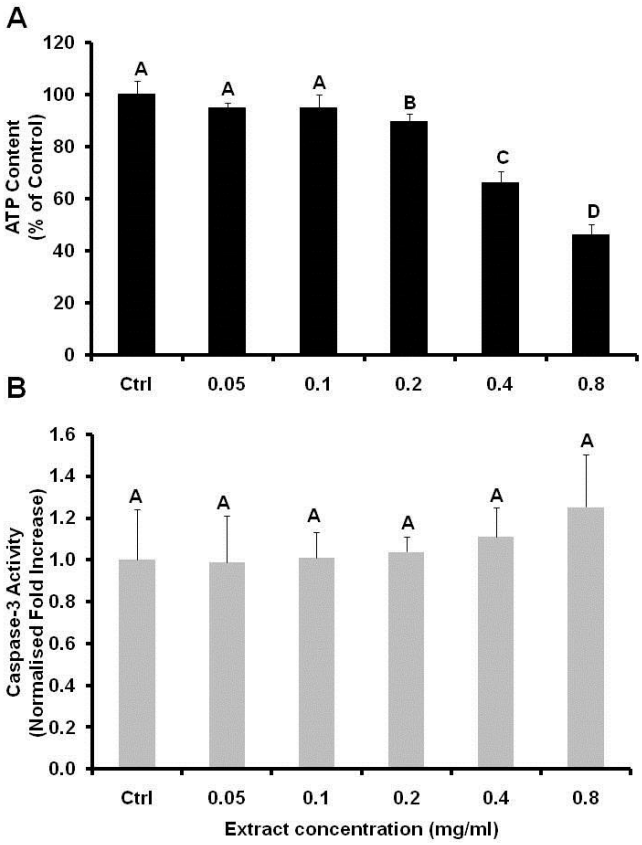

Fig. 5.2 Effect of green tea on cell viability (A) and apoptosis (B) of macrophages after 24 hrs. Different uppercase letters indicate significant differences ($P<0.05$) when compared to control treatment.

(ii) Rooibos

Treatment of the macrophages with rooibos extract for 24 hrs, decreased cell viability significantly ($P<0.05$) in a dose dependent manner from 0.05 mg extract/ml and higher concentrations (Fig. 5.3A). However, no significant effect on the induction of apoptosis was detected, although an increase (not significantly) was observed at the higher concentrations (Fig. 5.3B).

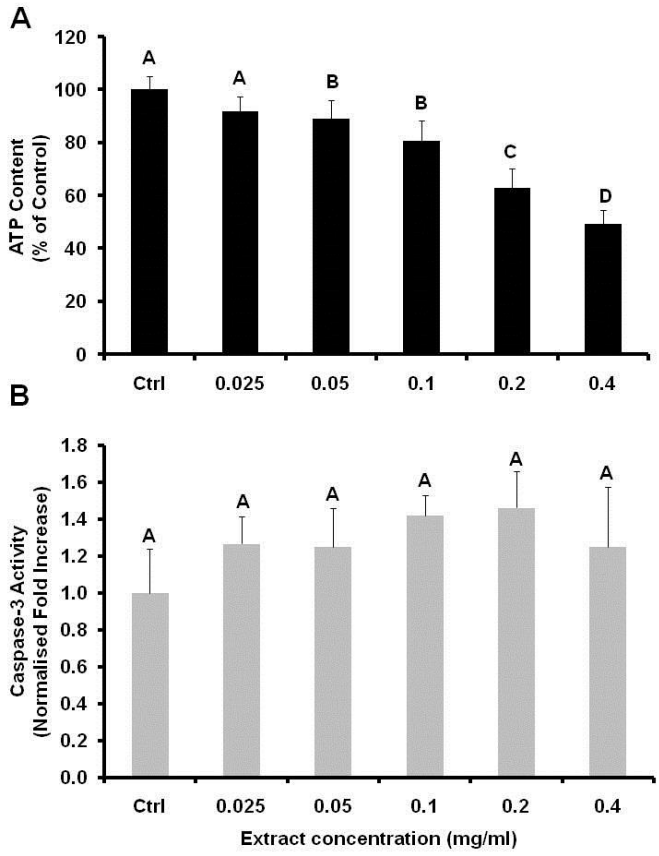

Fig. 5.3 Effect of rooibos on cell viability (A) and apoptosis (B) of macrophages after 24 hrs. Different uppercase letters indicate significant differences ($P<0.05$) when compared to control treatment.

(iii) Cyclopia subternata

Cyclopia subternata also significantly ($P<0.05$) decreased the viability of the macrophages after 24 hrs in a dose dependent manner (Fig. 5.4A). The capase-3 activity was not significantly increased (Fig. 5.4B).

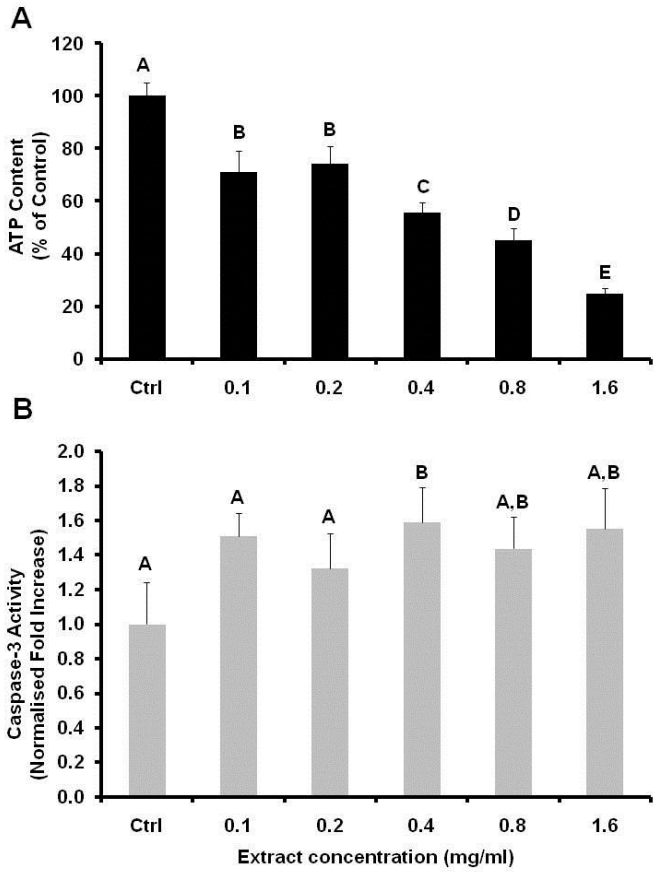

Fig. 5.4 Effect of *C. subternata* on cell viability (A) and apoptosis (B) of macrophages after 24 hrs. Different uppercase letters indicate significant differences ($P<0.05$) when compared to control treatment.

(iv) Cyclopia maculata

Cyclopia maculata extract decreased the viability of the macrophages significantly in a dose-dependent manner (Fig. 5.5A). The caspase-3 activity was increased significantly ($P<0.05$) at some of the higher concentrations (0.25 and 1 mg extract/ml) of *C. maculata,* while no significant effect was detected at the lower concentrations (Fig. 5.5B).

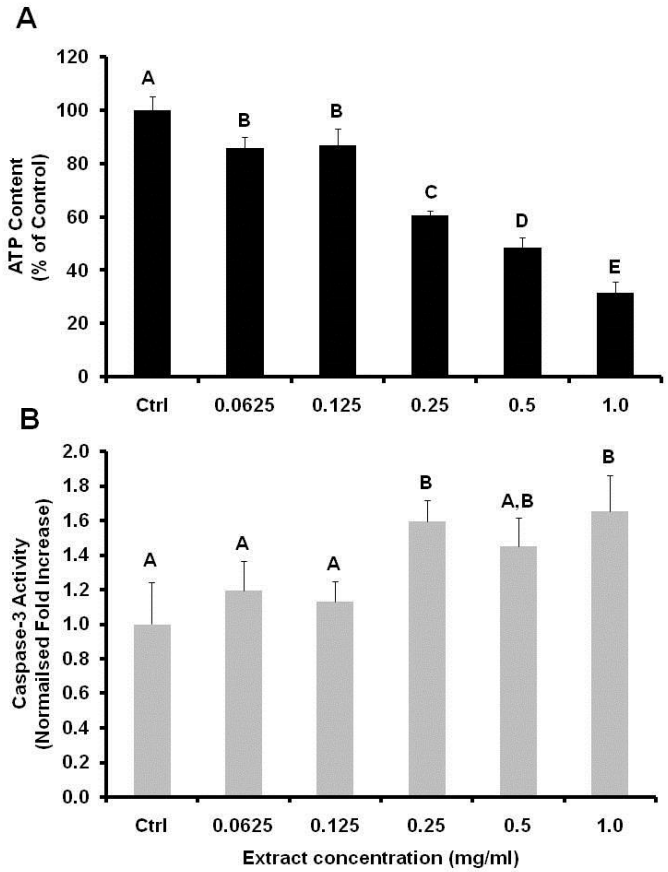

Fig. 5.5 Effect of *C. maculata* on cell viability (A) and apoptosis (B) of differentiated macrophages after 24 hrs. Different uppercase letters indicate significant differences ($P<0.05$) when compared to control (fresh culture medium).

(v) IC_{50} values for extract concentrations against macrophage viability

The rooibos extract required the lowest concentration to achieve a 50% reduction in cell viability followed by green tea, *C. maculata* and *C. subternata*. However, the effect of *C.maculata* on cell viability did not differ significantly from *C. subternata* (Table 5.5).

Table 5.6 IC_{50} values calculated from basal cell viability data

Extract	IC_{50} values (mg/ml)
Green tea	0.42 ± 0.04^{a}
Rooibos	0.19 ± 0.11^{b}
C. subternata	1.00 ± 0.44^{c}
C. maculata	0.64 ± 0.27^{ac}

Values present mean ± standard deviation; different lowercase letters in superscript indicate differences ($P<0.05$) between IC_{50} values.

5.3.3.2. Modulation of cell survival and anti-inflammatory indices by the tea and herbal tea extracts in the pre-exposure inflammatory model

(i) Effects of green tea extract on cell viability, TNF-α release and apoptosis

In the pre-exposure inflammatory model, green tea decreased ($P<0.05$) cell viability following the cytokine-inducing LPS stimulation by up to approximately 40% at the two highest concentrations of the extract used (Table 5.6). TNF-α release, both direct and normalised relative to cell viability, was decreased in a dose dependent manner with the highest decrease associated with a reduction in cell viability. However, up to 50% TNF-α was detected at the lowest extract concentration, with no significant effect on cell viability. Caspase-3 activity (fold increase) showed no direct increase, although when analysed relative to cell viability, an extract concentration of 0.2 mg/ml and higher showed an apparent 2-fold increase ($P<0.05$).

(ii) Effects of rooibos extract on cell viability, TNF-α release and apoptosis

A significant decrease in cell viability occurred only at the highest rooibos concentration of 0.4 mg extract/ml (Table 5.6). TNF-α release was reduced significantly ($P<0.05$), both directly, normalised according to cell viability and % only at the two highest extract concentrations. No significant effect by any of

the extract concentrations was noticed on caspase-3 activity (fold increase) directly or when normalised to cell viability.

(iii) Effects of C. subternata extract on cell viability, TNF-α release and apoptosis

The pre-exposure of the macrophages to *C. subternata* resulted in a significant decrease ($P<0.05$) in cell viability at 0.2 mg extract/ml and higher concentrations (Table 5.7). TNF-α release was decreased significantly ($P<0.05$) when considering the direct and normalised data and % TNF-α compared to the control at 0.1 mg extract/ml and at higher concentrations. Pre-exposure did not affect caspase-3 activity (fold increase) directly or when normalised to cell viability.

(iv) Effects of C. maculata extract on cell viability, TNF-α release and apoptosis

Cyclopia maculata decreased cell viability only at 1 mg extract/ml (Table 5.7). TNF-α release was also decreased when considering the direct, normalised data according to cell viability and the %, significantly ($P<0.05$) at all the extract concentrations. Caspase-3 activity (fold increase), direct and normalised to cell viability was not affected at an extract concentration of 0.063 mg/ml, while it decreased at higher concentrations of the extract.

Table 5.6 Effect of pre-exposure to green tea and rooibos extracts on cell viability and TNF-α release of THP-1 derived macrophages

Biological Endpoints	Negative Control	Positive Control	Green tea (mg/ml)				
			0.05	0.1	0.2	0.4	0.8
ATP content (% of positive control)	103.0 ± 19.9^{A}	$100.0\pm15.3^{A}{}_{a}$	$81.1 \pm 10.8_{a}$	$85.8 \pm 6.2_{a}$	$86.4 \pm 10.3_{a}$	$62.7\pm5.3_{b}$	$56.1\pm5.9_{b}$
TNF-α (pg/ml)	43.5 ± 9.2^{A}	$348.9\pm31.1^{B}{}_{a}$	$129.6\pm19.7_{b}$	$78.0\pm6.8_{c}$	$77.6\pm12.7_{c}$	$71.1\pm15.5_{c}$	$37.1\pm2.0_{d}$
Normalised TNF-α* (pg/ml)	42.3 ± 9.0^{A}	$348.9\pm31.1^{B}{}_{a}$	$188.3\pm28.7_{b}$	$110.2\pm9.7_{c}$	$115.2\pm18.8_{c}$	$113.3\pm24.6_{c}$	$66.2\pm3.5_{d}$
TNF-α**(% of positive control)	12.1 ± 2.6^{A}	$100.0\pm8.9^{B}{}_{a}$	$54.0\pm8.2_{b}$	$31.6\pm2.8_{c}$	$33.0\pm5.4_{c}$	$32.5\pm7.1_{c}$	$19.0\pm1.0_{d}$
Caspase-3 Fold Increase	0.5 ± 0.1^{A}	$1.0\pm0.2^{B}{}_{a}$	$0.8\pm0.1_{a}$	$1.0\pm0.1_{a}$	$1.3\pm0.3_{a}$	$1.2\pm0.2_{a}$	$1.2\pm0.1_{a}$
Normalised Caspase-3 Fold Increase***	0.5 ± 0.1^{A}	$1.0\pm0.2^{B}{}_{a}$	$1.1\pm0.1_{a}$	$1.4\pm0.2_{a}$	$1.9\pm0.4_{b}$	$1.9\pm0.4_{b}$	$2.1\pm0.2_{b}$
Biological Endpoints	**Negative Control**	**Positive Control**	**Rooibos (mg/ml)**				
			0.025	**0.05**	**0.1**	**0.2**	**0.4**
ATP content (% of positive control)	103.0 ± 19.9^{A}	$100.0\pm15.3^{A}{}_{a}$	$85.2\pm10.4_{a}$	$84.0\pm9.1_{a}$	$94.1\pm14.1_{a}$	$83.9\pm6.9_{a}$	$79.5\pm8.4_{a}$
TNF-α (pg/ml)	43.5 ± 9.2^{A}	$348.9\pm31.1^{B}{}_{a}$	$242.7\pm70.9_{a}$	$221.5\pm60.8_{a}$	$331.6\pm60.7_{a}$	$186.5\pm87.6_{b}$	$144.7\pm39.4_{b}$
Normalised TNF-α* (pg/ml)	42.3 ± 9.0^{A}	$348.9\pm31.1^{B}{}_{a}$	$284.9\pm83.2_{a}$	$261.2\pm71.7_{a}$	$437.4\pm80.0_{a}$	$222.4\pm10.5_{b}$	$172.4\pm46.9_{b}$
TNF-α**(% of positive control)	12.1 ± 2.6^{A}	$100.0\pm8.9^{B}{}_{a}$	$81.7\pm23.8_{a}$	$74.9\pm20.5_{a}$	$125.4\pm22.9_{a}$	$63.7\pm29.9_{b}$	$49.4\pm13.4_{b}$
Caspase-3 Fold Increase	0.5 ± 0.1^{A}	$1.0\pm0.2^{B}{}_{a}$	$1.3\pm0.2_{a}$	$1.1\pm0.2_{a,}$	$1.0\pm0.1_{a}$	$1.1\pm0.1_{a}$	$0.9\pm0.1_{a}$
Normalised Caspase-3 Fold Increase***	0.5 ± 0.1^{A}	$1.0\pm0.2^{B}{}_{a}$	$1.5\pm0.2_{b}$	$1.3\pm0.2_{a,b}$	$1.3\pm0.1_{a,b}$	$1.3\pm0.1_{a,b}$	$1.1\pm0.1_{a,b}$

Macrophages were incubated for 24 hrs in fresh medium containing the specified concentrations of tea extracts following incubation with the 10 ng/ml LPS for 6 hrs. Values represent mean ± standard deviation of at least five replications. Different uppercase letters in superscript in a row indicate differences between negative control and positive control; Different lowercase letters in subscript in a row indicate differences between the different concentrations of tea extracts and positive control. * Normalised TNF-α was calculated by correcting the TNF-α according to cell viability, **% TNF-α is the percentage of positive control TNF-α release. ***Normalised Caspase-3 Fold Increase is the Caspase-3 fold increase normalised by using the percentage ATP content. Statistical significance was considered at $P < 0.05$.

Table 5.7 Effect of pre-exposure to *C. subternata* and *C. maculata* extracts on cell viability and TNF-α release on THP-1 derived macrophages

Biological Endpoints	Negative Control	Positive Control	*C. subternata* (mg/ml)				
			0.1	**0.2**	**0.4**	**0.8**	**1.6**
ATP content (% of positive control)	103.0 ± 19.9^{A}	$100.0\pm15.3^{A}{}_{a}$	$92.7\pm9.4_{a}$	$79.5\pm4.9_{b}$	$72.4\pm5.6_{b}$	$72.7\pm3.9_{b}$	$47.9\pm6.4_{c}$
TNF-α (pg/ml)	43.5 ± 9.2^{A}	$348.9\pm31.1^{B}{}_{a}$	$165.7\pm25.1_{b}$	$78.2\pm15.5_{c}$	$80.8\pm11.0_{c}$	$140.9\pm4.7_{b}$	$55.3\pm2.9_{c}$
Normalised TNF-α* (pg/ml)	42.3 ± 9.0^{A}	$348.9\pm31.1^{B}{}_{a}$	$178.7\pm27.1_{b}$	$98.3\pm19.6_{c}$	$111.7\pm15.1_{c}$	$193.9\pm6.5_{b}$	$115.5\pm6.1_{c}$
TNF-α(% of positive control)**	12.1 ± 2.6^{A}	$100.0\pm8.9^{B}{}_{a}$	$51.2\pm7.8_{b}$	$28.2\pm5.6_{c}$	$32.0\pm4.3_{c}$	$55.6\pm1.9_{b}$	$33.1\pm1.8_{c}$
Caspase-3 Fold Increase	0.5 ± 0.1^{A}	$1.0\pm0.2^{B}{}_{a}$	$0.6\pm0.1_{b}$	$0.8\pm0.1_{a,b}$	$0.8\pm0.1_{a,b}$	$0.9\pm0.2_{a,b}$	$0.7\pm0.2_{a,b}$
Normalised Caspase-3 Fold Increase***	0.5 ± 0.1^{A}	$1.0\pm0.2^{B}{}_{a}$	$0.7\pm0.1_{a}$	$1.0\pm0.1_{a}$	$1.1\pm0.1_{a,b}$	$1.2\pm0.2_{a,b}$	$1.5\pm0.4_{b}$
Biological Endpoints	**Negative Control**	**Positive Control**	***C. maculata* (mg/ml)**				
			0.063	**0.13**	**0.25**	**0.5**	**1.0**
ATP content (% of positive control)	103.0 ± 19.9^{A}	$100.0\pm15.3^{A}{}_{a}$	$86.8\pm12.9_{a}$	$80.2\pm12.3_{a}$	$86.0\pm10.6_{a,b}$	$81.5\pm7.3a_{b}$	$71.4\pm12.2_{b}$
TNF-α (pg/ml)	43.5 ± 9.2^{A}	$348.9\pm31.1^{B}{}_{a}$	$78.7\pm13.8_{b}$	$86.4\pm11.9_{b}$	$67.0\pm12.3_{b}$	$83.0\pm9.5_{b}$	$55.1\pm10.8_{b}$
Normalised TNF-α* (pg/ml)	42.3 ± 9.0^{A}	$348.9\pm31.1^{B}{}_{a}$	$90.6\pm15.9_{b}$	$107.8\pm14.9_{b}$	$77.9\pm14.3_{b}$	$101.8\pm11.6_{b}$	$77.1\pm15.1_{b}$
TNF-α(% of positive control)**	12.1 ± 2.6^{A}	$100.0\pm8.9^{B}{}_{a}$	$26.0\pm4.6_{b}$	$30.9\pm4.3_{b}$	$22.3\pm7.3_{b,c}$	$29.2\pm3.3_{c}$	$22.1\pm4.3_{c}$
Caspase-3 Fold Increase	0.5 ± 0.1^{A}	$1.0\pm0.2^{B}{}_{a}$	$0.7\pm0.1_{a}$	$0.5\pm0.0_{b}$	$0.6\pm0.1_{b}$	$0.6\pm0.1_{b}$	$0.5\pm0.0_{b}$
Normalised Caspase-3 Fold Increase***	0.5 ± 0.1^{A}	$1.0\pm0.2^{B}{}_{a}$	$0.8\pm0.1_{a}$	$0.7\pm0.1_{b}$	$0.7\pm0.1_{b}$	$0.7\pm0.1_{b}$	$0.7\pm0.1_{b}$

Macrophages were incubated for 24 hrs in fresh medium containing the specified concentrations of tea extracts and thereafter incubated with the 10 ng/ml LPS for 6 hrs. Values represent mean ± standard deviation of at least five replications. Different uppercase letters in superscript in a row indicate differences between negative control and positive control; different lowercase letters in subscript in a row indicate differences between the different concentrations of tea extracts and positive control. * Normalised TNF-α was calculated by correcting the TNF-α according to cell viability. **% TNF-α is the percentage of positive control TNF-α release. ***Normalised Caspase-3 Fold Increase is the Caspase-3 fold increase normalised by using the percentage ATP content. Statistical significance was considered at $P < 0.05$.

5.3.3.3. *Modulation of cell survival and anti-inflammatory indices by the tea and herbal tea extracts in the co-exposure inflammatory model*

(i) Effects of green tea extracts on cell viability, TNF-α release and apoptosis

In the co-exposure inflammatory model, green tea significantly ($P<0.05$) decreased cell viability in the presence of LPS, lowering the ATP content to approximately 50% at the highest concentrations of the extract used. Co-exposure decreased TNF-α release, directly, normalised to the cell viability and the % release, exhibiting a dose response effect. The direct and normalised caspase-3 activities were increased significantly (up to 4-fold) at 0.4 and 0.8 mg/ml extract concentrations.

(ii) Effects of rooibos extracts on cell viability, TNF-α release and apoptosis

A significant ($P<0.05$) decrease in cell viability occurred at the highest extract concentration (0.4 mg /ml) only. Co-exposure with LPS also showed a significant decrease ($P<0.05$) in the direct TNF-a release, while normalised TNF release was decreased significantly only at 0.1 mg/ml and % of TNF-α at the two highest extract concentrations. A significant increase ($P<0.05$) in the direct and normalised caspase-3 activity was detected at the two highest concentrations of extract.

(iii) Effects of C. subternata *extracts on cell viability, TNF-α release and apoptosis*

The co-exposure of the macrophages to *C. subternata* resulted in a significant decrease ($P<0.05$) of cell viability at a concentration of 0.2 mg/ml and above. A significant decrease ($P<0.05$) in TNF-α was detected at all concentrations of the extract regarding the direct, normalised and % of TNF-α release. The direct caspase-3 activity displayed an increase ($P<0.05$) in caspase-3 activity (fold increase) from 0.8 mg/ml and normalised caspase 3 activity from 0.4mg/ml and above.

(iv) Effects of C. maculata *extracts on cell viability, TNF-α release and apoptosis*

The *C. maculata* extract also significantly decreased ($P<0.05$) cell viability at the two highest concentrations. The direct, normalised and % of TNF-α release was also decreased significantly ($P<0.05$) by all the extract concentrations exhibiting a clear dose response. Co-exposure of the extract with LPS, however, significantly increased the caspase-3 activity (normalised only) at the three highest extract concentrations.

Table 5.8 Effect of green tea and rooibos extracts on cell viability, TNF-α release and apoptosis when co-exposed with LPS.

Biological Endpoints	Negative Control	Positive Control	Green tea (mg/ml)				
			0.05	0.1	0.2	0.4	0.8
% ATP content	94.2 ± 7.8^{A}	$100.0\pm14.4^{A}_{a}$	$97.4\pm15.5_{a}$	$95.1\pm11.5_{a}$	$86.6\pm11.6_{b}$	$71.4\pm12.1_{c}$	$50.6\pm9.2_{d}$
TNF-α (pg/ml)	33.0 ± 11.5^{A}	$204.4\pm36.4^{B}_{a}$	$60.2\pm9.0_{b}$	$50.8\pm9.4_{b}$	$24.6\pm6.6_{c}$	$19.5\pm5.4_{c}$	$14.2\pm2.1_{c}$
Normalised TNF-α* (pg/ml)	23.2 ± 9.6^{A}	$204.4\pm36.4^{B}_{a}$	$61.8\pm9.3_{b}$	$53.4\pm9.8_{b}$	$28.4\pm7.6_{b}$	$27.3\pm7.6_{b}$	$28.1\pm4.2_{b}$
% TNF-α**	17.1 ± 6.0^{A}	$100.1\pm17.8^{B}_{a}$	$30.2\pm4.5_{b}$	$26.1\pm4.8_{b}$	$13.9\pm3.7_{b,c}$	$13.3\pm3.7_{c}$	$13.8\pm2.0_{b,c}$
Caspase-3 Fold Increase	1.0 ± 0.1^{A}	$1.3\pm0.3^{A}_{a}$	$1.5\pm0.3_{a}$	$1.6\pm0.2_{a}$	$1.6\pm0.2_{a}$	$2.4\pm0.2_{b}$	$2.2\pm0.2_{b}$
Normalised Caspase-3 Fold Increase***	1.0 ± 0.1^{A}	$1.3\pm0.3^{A}_{a}$	$1.5\pm0.3_{a}$	$1.7\pm0.2_{a}$	$1.8\pm0.2_{a}$	$3.4\pm0.2_{b}$	$4.4\pm0.4_{c}$
Biological Endpoints	**Negative Control**	**Positive Control**	**Rooibos (mg/ml)**				
			0.025	**0.05**	**0.1**	**0.2**	**0.4**
% ATP content	94.2 ± 7.8^{A}	$100.0\pm14.4^{A}_{a}$	$100.2\pm8,6_{a}$	$91.1\pm6.8_{a}$	$99.8\pm15.8_{a}$	$89.1\pm12.9_{a}$	$74.6\pm10.8_{b}$
TNF-α (pg/ml)	33.0 ± 11.5^{A}	$204.4\pm36.4^{B}_{a}$	$198.6\pm33.4_{a}$	$148.3\pm27.8_{b}$	$134.4\pm17.4_{b}$	$109.9\pm9.6_{b}$	$110.0\pm18.1_{b}$
Normalised TNF-α* (pg/ml)	23.2 ± 9.6^{A}	$204.4\pm36.4^{B}_{a}$	$198.6\pm33.4_{a}$	$162.9\pm30.5_{a,b}$	$134.7\pm17.4_{b}$	$123.4\pm10.8_{a,b}$	$147.5\pm24.2_{a,b}$
% TNF-α**	17.1 ± 6.0^{A}	$100.1\pm17.8^{B}_{a}$	$97.2\pm14.9_{a}$	$79.7\pm15.3_{a}$	$65.9\pm8.5_{a,b}$	$60.4\pm5.3_{b}$	$72.2\pm11.8a,_{b}$
Caspase-3 Fold Increase	1.0 ± 0.1^{A}	$1.3\pm0.3^{A}_{a}$	$1.6\pm0.1_{a}$	$1.5\pm0.3_{a}$	$1.5\pm0.2_{a}$	$1.9\pm0.2_{b}$	$2.0\pm0.4_{b}$
Normalised Caspase-3 Fold Increase***	1.0 ± 0.1^{A}	$1.3\pm0.3^{A}_{a}$	$1.6\pm0.1_{a}$	$1.7\pm0.3_{a}$	$1.5\pm0.2_{a}$	$2.2\pm0.2_{b}$	$2.7\pm0.5_{b}$

Rested macrophages were incubated with 10 ng/ml LPS and the specified tea extract concentrations for 6 hrs. Values represent mean ± standard deviation of at least five replications. Uppercase letters in superscript in a row indicate differences between negative control (no LPS, no anti-inflammatory compounds) and positive control (LPS, no anti-inflammatory compounds); Different lowercase letters in subscript in a row indicate differences between the different concentrations of tea extracts and positive control. * Normalised TNF-α was calculated by correcting the TNF-α according to cell viability, **% TNF-α is the percentage of positive control TNF-α release. ***Normalised Caspase-3 Fold Increase is the Caspase-3 fold increase normalised by using the percentage ATP content. Statistical significance was considered at $P < 0.05$.

Table 5.9 Effect of *C. subternata* and *C. maculata* extracts on cell viability, TNF-α release and apoptosis when co-exposed with LPS.

Biological Endpoints	Negative Control	Positive Control	*C. subternata* (mg/ml)				
			0.1	0.2	0.4	0.8	1.6
% ATP content	94.2 ± 7.8^{A}	$100.0\pm14.4^{A}_{a}$	$93.3\pm11.5_{a}$	$85.5\pm8.3_{b}$	$72.7\pm9.2_{b}$	$62.1\pm10.2_{b}$	$34.2\pm10.8_{c}$
TNF-α (pg/ml)	33.0 ± 11.5^{A}	$204.4\pm36.4^{B}_{a}$	$83.7\pm10.9_{b}$	$67.6\pm11.9_{b}$	$50.5\pm2.0_{c}$	$47.3\pm4.4_{c}$	$55.4\pm6.6_{c}$
Normalised TNF-α* (pg/ml)	23.2 ± 9.6^{A}	$204.4\pm36.4^{B}_{a}$	$89.7\pm11.7_{b}$	$79.1\pm14.0_{b}$	$69.5\pm2.8_{c}$	$76.2\pm7.0_{c}$	$49.5 \pm 17.5_{d}$
% TNF-α**	17.1 ± 6.0^{A}	$100.1\pm17.8^{B}_{a}$	$43.9\pm5.7_{b}$	$38.7\pm6.8_{b}$	$34.0\pm1.4_{c}$	$37.3\pm3.4_{c}$	$25.7 \pm 8.8_{c}$
Caspase-3 Fold Increase	1.0 ± 0.2^{A}	$1.3\pm0.4^{A}_{a}$	$1.5\pm0.2_{a}$	$1.4\pm0.1_{a}$	$1.7\pm0.1_{a}$	$1.9\pm0.1_{b}$	$1.4\pm0.5_{a}$
Normalised Caspase-3 Fold Increase***	1.0 ± 0.2^{A}	$1.3\pm0.4^{A}_{a}$	$1.7\pm0.2_{a}$	$1.6\pm0.2_{a}$	$2.3\pm0.2_{b}$	$3.0\pm0.1_{c}$	$4.1\pm0.7_{d}$
	Negative Control	Positive Control	*C. maculata* (mg/ml)				
			0.063	0.13	0.25	0.5	1.0
% ATP content	94.2 ± 7.8^{A}	$100.0\pm14.4^{A}_{a}$	$102.8\pm13.3_{a}$	$92.3\pm10.7_{a}$	$82.3\pm21.8_{a,b}$	$70.2\pm11.2_{b}$	$52.8\pm15.4_{b}$
TNF-α (pg/ml)	33.0 ± 11.5^{A}	$204.4\pm36.4^{B}_{a}$	$94.4\pm17.9_{b}$	$85.9\pm9.5_{b}$	$51.4\pm9.2_{c}$	$35.8\pm4.0_{c}$	$30.6\pm3.9_{c}$
Normalised TNF-α* (pg/ml)	23.2 ± 9.6^{A}	$204.4\pm36.4^{B}_{a}$	$91.9\pm17.4_{b}$	$97.1\pm10.8_{b}$	$59.6\pm10.7_{c}$	$51.0\pm5.6_{c}$	$58.0\pm7.3_{c}$
% TNF-α**	17.1 ± 6.0^{A}	$100.1\pm17.8^{B}_{a}$	$44.9\pm8.5_{b}$	$47.5\pm5.3_{b}$	$29.2\pm5.2_{c}$	$25.0\pm2.8_{c}$	$28.4\pm3.6_{c}$
Caspase-3 Fold Increase	1.0 ± 0.2^{A}	$1.3\pm0.4^{A}_{a}$	$1.3\pm0.3_{a}$	$1.5\pm0.1_{a}$	$1.9\pm0.1_{b}$	$2.3\pm0.2_{b}$	$1.7\pm0.3_{ab}$
Normalised Caspase-3 Fold Increase***	1.0 ± 0.2^{A}	$1.3\pm0.4^{A}_{a}$	$1.3\pm0.4_{a}$	$1.8\pm0.1_{a}$	$2.4\pm0.2_{b}$	$3.5\pm0.4_{c}$	$3.4\pm0.7_{c}$

Rested macrophages were incubated with 10 ng/ml LPS and the specified tea extract concentrations for 6 hrs. Values represent mean ± standard deviation of at least five replications. Different uppercase letters in superscript in a row indicate differences between negative control and positive control; Different lowercase letters in subscript in a row indicate differences between the different concentrations of tea extracts and positive control. * Normalised TNF-α was calculated by correcting the TNF-α according to cell viability. **% TNF-α is the percentage of positive control TNF-α release. ***Normalised Caspase-3 Fold Increase is the Caspase-3 fold increase normalised by using the percentage ATP content. Statistical significance was considered at $P < 0.05$.

5.4. Discussion

Chronic inflammation has been suggested to be involved in cancer development (Mantovani et al., 2008; Medzhitov, 2010; Mueller, 2006; Multhoff et al., 2011) and has become a potential target for the prevention of cancer using natural products. A major role of macrophages and TNF-α release in acute and chronic inflammation has been defined (Aggarwal et al., 2006). Macrophages play a pivotal role in the inflammatory process with their participation in antigen processing and presentation to antigen specific T-cells (Cavaillon, 1994). Upon stimulation by inflammatory stimuli, such as LPS, these immune cells modulate the expression of toll-like receptor 4 (TLR4). This, in turn, leads to the activation of the NF-κB and mitogen-activate protein kinase (MAPK) pathways. The translocation of free NF-κB to the nucleus induces the transcription of pro-inflammatory mediators, such as inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), TNF-α, IL-1β, -6 and -8 (Kim et al., 2008). Of these, TNF- α, is one of the classical macrophage acute phase cytokines that is produced in response to external stress such as LPS (Tsukamoto, 2002).

Non-steroidal anti-inflammatory drugs, such as dexamethasone and ibuprofen, modulate the inflammatory response by inhibiting the expression of COX-2, which leads to the inhibition of prostaglandin production, also a potent pro-inflammatory mediator (Vane and Botting, 1998). Plant flavonoids have displayed anti-inflammatory activities in many *in vitro* models. The mechanisms suggested to be involved in this action include the inhibition of NF-κB, COX-2 and iNOS (Serafini et al., 2010). Several flavonoids have also been shown to have the ability to decrease the expression of different pro-inflammatory cytokines in RAW macrophages, Jurkat T-cells and peripheral blood mononuclear cells by mediating NF-κB inhibition and the reduction of the MAPK activity related to cell proliferation (Santangelo et al., 2007).

The focus of the current study was on the development of an *in vitro* macrophage cell culture inflammatory model and the screening of the anti-inflammatory effects of extracts of green tea, rooibos and two *Cyclopia* spp. extracts utilizing a pre- and co-exposure approach on the reduction of TNF-α release. The

modulating role of the different plant extracts was critically assessed in relation to the effect on cell viability, apoptosis and the inhibition of TNF-α release.

The green tea and herbal tea extracts exhibited a dose response effect on cell viability without overtly affecting apoptosis, except for the *C. maculata* and *C. subternata* extract, which increased apoptosis significantly at the highest experimental concentration after pre-exposure of the macrophages for 24 hrs. The modulation of cell viability could be ascribed to the respective polyphenol constituents of the different plant extracts which varies with respect to their antioxidant potency as discussed in Chapter 3. The effect on cell viability has been related to the disruption of the mitochondrial membrane potential due to polyphenol membrane interactions involving Fe (II) resulting in increased lipid peroxidation (Magcwebeba, 2013). In order to investigate anti-inflammatory effects of the plant extracts against cytokine release, care should therefore be taken to avoid the masking effects of the disruption of cell growth parameters, which in the case of green tea, led to induction of the apoptosis at highest dose level utilised.

The green tea extract displayed a significant ($P<0.05$) reducing effect on the release of TNF-α in the pre-exposure model at all the extract concentrations. Cell viability was reduced only at the two highest extract concentrations whilst direct apoptosis tend to remain unaffected. It would appear that a “memory effect” seems to exist, culminating in an interaction between LPS exposure and the green tea polyphenols inhibiting TNF-α release. Removal of THP-1 derived macrophages via apoptosis is therefore not responsible for the reduction in TNF-α release at the lower extract concentrations. At higher extract concentrations, the reduction in cell viability could play a role and could result from an increase oxidative stress due to an interaction between the green tea polyphenols and the production to inflammatory mediators related to LPS exposure (Kallapura et al., 2014). This can be attributed to the most prevalent green tea polyphenol, EGCG, which is known to be a potent antioxidant likely to exhibit pro-oxidant behaviour under the current experimental conditions. However, the decreased TNF-α release by the green tea extract, specifically at the lower concentrations could be related to direct inhibition via a different pathways related to the suppression of inflammatory pathway of TNF-α release as reported for EGCG (Gupta et al., 2014). However other catechins could

also play an additive and/or synergistic role in combination with EGCG. In the co-exposure model TNF-a release was also inhibited but to a greater extend which was not only associated with a decrease in cell viability but also an increase in apoptosis. It would appear that the combined effect of LPS and green tea extract is likely to enhanced oxidative stress in the macrophages due to increased pro-oxidant properties of the flavanols and the inflammatory mediators induced by LPS. The co-exposure macrophage model provides a less sensitive approach to investigate the anti-inflammatory effect of green tea.

The rooibos extract decreased TNF-α in the pre-exposure model at the two highest concentrations of the extract, while cell viability was affected at the highest concentration with apoptosis not affected. The rooibos extract displays a lower anti-oxidant activity in a lipid environment than green tea (Chapter 3) which could be ascribed to an higher hydrophilic nature of the rooibos flavonoids, such as aspalathin, in comparison to the green tea catechin, EGCG (Snijman et al., 2009). As lower extract concentrations were utilised compared to green tea, the memory effect was also associated with the reduction of TNF-α release. A weaker response was obtained when compared to green tea at similar extract concentration, once again pointing to a decreased activity of the rooibos flavanoids in lipid environments. A slight increase inhibitory effect of the rooibos extract on TNF-α release was detected in the co-exposure model which again could be ascribed to increased oxidative stress associated with a decrease in cell viability and increase in apoptosis.

Both *Cyclopia* species reduced the release of TNF-α more effectively during the pre-exposure model than in the co-exposure model. In the pre-exposure model the reduction of TNF-α is associated with a decrease in cell viability, especially at the higher concentration whilst no apoptosis were detected. Of interest is that *C. maculata* exhibited a higher protection against TNF-α release as compared to *C. subternata*. The higher mangiferin level is likely to be responsible for the higher protective ability as it is known to exhibited anti-inflammatory effects (Gong et al., 2013). In the co-exposure model the reduction in TNF-α is associated with an increased level of apoptosis which, as described for green tea and rooibos, likely to

remove cells containing high levels of TNF-α. The interaction of the *Cyclopia* extracts with the inflammatory mediators induced by LPS may increase oxidative stress resulting in apoptosis. It is known that under certain conditions, depending on the concentration, mangiferin also exhibits pro-oxidative activity (Pardo-Andreu et al., 2007). As discussed for green tea and rooibos extracts, the interaction of the *Cyclopia* extracts with the inflammatory mediators induced by LPS may increase oxidative stress leading to apoptosis. However, the anti-inflammatory effect of both species at lower extract concentrations in both the pre- and co-exposure inflammatory models may be attributed to the anti-inflammatory effect of mangiferin (Gong et al., 2013), the most prevalent flavonoid in *C. subternata* and *C. maculata.* It was also demonstrated by Bumrungpert et al. (2010) that xanthones, possess the ability to inhibit LPS-induced inflammation in human macrophages, by reducing the expression of certain inflammatory genes. Mangiferin is also known to be less hydrophobic than EGCG and aspalathin and needs the presence of much higher concentrations to exhibit the desired effect (Chapter 3), as evident from the concentrations used of the *Cyclopia spp*. in this study.

Findings of the current study suggest that pre-exposure provide a better approach to explore the anti-inflammatory properties of the herbal tea extracts as co-exposure could resulted in increased oxidative stress parameters leading to apoptosis. The importance of NFκB expression and translocation in mediating the inflammation process still has to be investigated to further elucidate the mechanism of the ability of the extracts to reduce TNF-α. Iron is known to play a critical role in macrophage-mediated cytotoxicity by contributing to the production of highly toxic hydroxyl radicals via the Fenton reaction and by controlling the production of nitric oxide (NO) after activation by immunologic stimuli (Mulero and Brock, 1999). A moderate increase in the nonheme iron content in hepatic marcrophages by erythrophagocytosis promotes subsequent LPS-stimulated NF-κB activation in a hemeoxygenase-dependent manner. Recent evidence also suggests a role of intracellular low molecular weight iron in the early signal transduction for LPS-mediated NF-κB activation (She et al., 2002; Tsukamoto, 2002; Xiong et al., 2003). The modulating role of polyphenol-iron interactions in TNF-α release is therefore of interest and need to be further explored.

Oxidative stress also plays an important role in the initiation of the immune response in macrophages. Upon stimulation with LPS the production of nitric oxide and other free radicals, such as superoxide anion, increases (Kallapura et al., 2014; Wu et al., 2006). It has been demonstrated that EGCG, a potent antioxidant from green tea, reduces the expression of NF-κB and subsequently reduces the release of TNF-α presumably through its antioxidant and radical scavenging properties (Yang et al., 1998). All the tea and herbal tea extracts tested exhibited varying antioxidant activities (Chapter 3), and it is possible that the anti-inflammatory activities may also be related to their ability to scavenge free radicals via different anti-oxidant mechanisms following LPS exposure and thereby reducing TNF-α release.

5.5. References

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Chapter 6

GENERAL DISCUSSION

General Discussion

Several studies have been conducted to investigate the anti-inflammatory effects of flavonoids and other polyphenolic constituents outlining possible mechanisms involved (Gonzalez et al., 2011; Rahman et al., 2006). Different molecular targets, such as the inhibition of cyclooxygenase, lipoxygenase and nuclear factor-kappaB (NF-κB) signaling resulting in the down regulation of the expression of pro-inflammatory markers in different cellular targets including macrophages, lymphocytes and epithelial cells have been identified. The structural variation and requirements with associated differences in antioxidant/radical scavenging properties have been associated with the biological and anti-inflammatory properties of polyphenols.

There is an abundance of anecdotal evidence that rooibos (*Aspalathus linearis*) and honeybush (*Cyclopia* spp.) can alleviate certain skin inflammatory conditions. The phenolic composition of these herbal teas has provided a scientific basis for the development of skin care products utilising extracts of these herbal teas (Joubert and de Beer, 2011; Joubert et al., 2008). Several studies have shown the anti-inflammatory and subsequent anti-tumour activities of rooibos and honeybush by the topical application to mouse skin (Marnewick et al., 2005; Petrova, 2009). The antioxidant activities of their polyphenolic compounds have also been shown as a major contributing factor to the biological properties and hypothesised to also play a role in their anti-inflammatory activity. In order to elucidate the possible anti-inflammatory activity of *C. maculata,* the present study investigated the comparative anti-inflammatory properties of aqueous extracts of unfermented rooibos, *C. subternata* and *C. maculata* in HaCaT skin keratinocytes and monocyte (THP-1) derived macrophages *in vitro*, utilising green tea (*Camellia sinensis*) as benchmark. The role of chemical constituents and antioxidant properties for assessing the anti-inflammatory properties of the extracts in skin cells and macrophages was evaluated.

For the screening of the anti-inflammatory effects of the herbal tea extracts in skin cells and macrophages, two *in vitro* inflammatory models were developed and

validated utilising known anti-inflammatory model compounds. The first model, a keratinocyte ultraviolet B (UVB) pre-exposure model, was modified from a previously developed post-exposure model screening the effects of green tea and herbal tea extracts following UVB exposure and the induction of interleukin (IL)-1α (Magcwebeba et al., 2012). In the present study the keratinocytes were exposed to green tea and different herbal tea extracts before exposure to UVB and the modulation of IL-1α monitored (Chapter 4). The second model, utilising THP-1 derived macrophages, was developed to screen the effect of the herbal tea extracts on the release of tumour necrosis factor alpha (TNF-α) following exposure to lipopolysaccharide (LPS). The effect of both the pre- and co-exposure treatments with green tea and the herbal extracts were investigated (Chapter 5).

Antioxidant properties

The overall low antioxidant activity of both honeybush extracts, when compared to green tea and rooibos, has been reported, which was aligned with the general notion that the major honeybush polyphenols exhibited a lower antioxidant activity (Joubert et al., 2008). Of interest is that, in the current study, the mangiferin and isomangiferin levels detected in *C. maculata* extract were 2-fold higher when compared to *C. subternata* extract. This species could therefore be a valuable commercial source for mangiferin, together with the other two xanthone-rich species, *C. longifolia* and *C. genistoides,* which was also shown to have high levels of mangiferin (Joubert et al., 2003; Magcwebeba, 2013). Except for the inhibition of lipid peroxidation, the extract of C*. maculata* exhibited the lowest antioxidant activity of the extracts monitored, including that of *C, subternata*. The high levels of the xanthones, mangiferin and iso-mangiferin, exhibiting a high redox potential, is likely to play an important protective role against cytotoxic effects while the hydrophobic nature also have been implicated in the limited antioxidant properties, specifically in lipid environments. Other differences when comparing the two honeybush spp. include the high level of hesperidin and low level of eriodictyol-glucoside and scolymoside in the *C. maculata* extract.

Keratinocyte UVB pre-exposure model

Exposure to UVB mainly penetrates into the epidermis and results in the generation of oxidative stress in keratinocytes, the predominant cells constituting this skin layer. The subsequent ROS mediated signal transduction stimulated pathways lead to the activation of activator protein-1 (AP-1) and NF-κB transcription factor complexes which regulate the release of important cytokines including interleukin-1 alpha (IL-1α) and tumor necrosis factor alpha (TNF-1α) (Kramer-Stickland et al., 1999). The herbal tea extracts displayed varying antioxidant activities (Chapter 3) and this may be a possible direct indication of the anti-inflammatory properties of the extracts. Various polyphenols have shown to have the ability to reduce UVB-induced inflammation by supressing the NF-κB and mitogen-activate protein kinase (MAPK) pathways (Afaq et al., 2003; Mantena and Katiyar, 2006; Vicentini et al., 2011; Weng et al., 2014) and this may be the targets for interferences from the herbal tea polyphenols in the current study. The release of iron from ferritin stores also participate in the UVB related oxidative damage and the activation of the abovementioned transcription factors related to the induction of inflammatory responses (Wagener et al., 2013). All the extracts showed the ability to reduce iron (III) to iron (II) and inhibited Fe(II)-induced lipid peroxidation. The stabilisation of iron, prohibiting the Fenton-type reaction by polyphenols, seems likely to also play a role in their anti-inflammatory properties.

The rooibos and green tea extracts exhibited a very similar anti-inflammatory response when considering the inhibition of IL-1α at the same extract concentration. However, the green extract tends to be more active at higher concentrations but it reduces cell viability and effect apoptosis which prohibited any comparisons. Of interest was that both honeybush extracts exhibited similar but slightly weaker anti-inflammatory effects compared to rooibos and green tea which could be ascribed to a lower antioxidant activities due to differences in the polyphenolic composition. Under the current experimental conditions no marked effects on cell viability or the induction of apoptosis were noticed which imply an anti-inflammatory effect. Therefore, the pro-oxidant effects of the polyphenolic constituents that mask the anti-inflammatory effects in the post-exposure model (Magcwebeba, 2013) did not play a major role in the pre-exposed UVB HaCaT cell model due to removal of the

polyphenol-containing culture media, thereby reducing their cellular levels facilitating their potential anti-inflammatory properties. However, the residual polyphenols absorbed into the keratinocytes may exhibit a protective sunscreen effect, lowering the vulnerability of the cells to the effects of UVB light that could result in a reduced inflammatory response, an aspect that should be further investigated in future.

THP-1/Macrophage model

Macrophages play a pivotal role in the inflammatory process with their participation in antigen processing and presentation to antigen specific T-cells (Cavaillon, 1994). Upon stimulation by inflammatory stimuli, such as LPS, these immune cells modulate the expression of toll-like receptor 4 (TLR4) which in turn, leads to the activation of the NF-κB and MAPK pathways. The translocation of free NF-κB to the nucleus induces the transcription of pro-inflammatory mediators, such as inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), TNF-α, IL-1β, IL-6 and IL-8 (Kim et al., 2008). Of these, TNF-α is one of the classical macrophage acute phase cytokines that is produced in response to external stress such as LPS (Tsukamoto, 2002). Plant flavonoids have displayed anti-inflammatory activities in many *in vitro* models. The mechanisms suggested to be involved include the inhibition of NF-κB, COX-2 and iNOS (Serafini et al., 2010). Several flavonoids have also been shown to have the ability to decrease the expression of different pro-inflammatory cytokines in RAW macrophages, Jurkat T-cells and peripheral blood mononuclear cells by mediating NF-κB inhibition and the reduction of the MAPK activity (Santangelo et al., 2007).

As described for the HaCaT model, green tea exhibited a higher anti-inflammatory effect than rooibos in the pre-exposure model with green tea exhibiting an increased apoptotic effect with a concomitant decrease in cell viability at higher concentrations. In contrast to the HaCaT model, where the two honeybush spp exhibited similar anti-inflammatory effect, *C. maculata* tend to exhibit a higher activity whilst it significantly reduced apoptosis at the highest extract concentration. Of interest is that the honeybush extracts exhibited a higher anti-inflammatory effect than rooibos at comparative concentrations, most likely due to the high xanthone

levels, specifically when considering the *C. maculata* extract. Co-exposure of the extracts with LPS provided a similar response with green tea, exhibiting the highest anti-inflammatory response although the induction of apoptosis and the reduction in cell viability became more prominent, presumably due to pro-oxidant effects. As described in the pre-exposure model, the extract of *C. maculata* was more effective with both honeybush extracts exhibiting a higher anti-inflammatory effect than the rooibos extract. As described for green tea, the role of apoptosis and reduction in cell viability became more prominent in the co-exposure model, masking the anti-inflammatory effect.

Several mechanism has been proposed whereby polyphenols could exhibit their anti-inflammatory effects (Gonzalez et al., 2011; Nichols and Katiyar, 2010). Oxidative stress plays an important role in the initiation of the immune response in macrophages. One of the important sources of reactive oxygen species (ROS) in macrophage is NADPH oxidase activation during phagocytosis. ROS regulate multiple cellular functions in macrophages via the formation of H_2O_2 that can affect various gene expressions, such as the activation of NF-κB and AP-1 which regulates the expression of multiple immune and inflammatory molecules (Aharoni-Simon et al., 2006; Hsu and Wen, 2002). Upon stimulation with LPS the production of nitric oxide and other free radicals, such as superoxide anion (Kallapura et al., 2014; Wu et al., 2006) increases. Tea and herbal tea extracts exhibited varying antioxidant activities, and it is possible that the anti-inflammatory activities may also be related to their ability to scavenge free radicals via different antioxidant mechanisms following LPS exposure and reduces TNF-α release.

It has been demonstrated that EGCG, a potent antioxidant from green tea, reduce the expression of NF-κB and subsequently reduces the release of TNF-α presumably through its antioxidant and radical scavenging properties (Yang et al., 1998). Though the anti-inflammatory effect of rooibos extracts is yet to be established, there are numerous studies supporting the ability of the polyphenolic constituents of rooibos to inhibit inflammation. The production and/or release of pro-inflammatory cytokines has been reduced by orientin-2”-O-galactopyranoside. This activity was attributed to the ability of the compound to inhibit the nuclear factor-κB

(NF-κB) and extracellular signal-regulated kinase (ERK) pathways in mouse brain cells (Zhou et al., 2014). Quercetin has shown anti-inflammatory activity against NF-κB activation (Hamalainen et al., 2007). Luteolin exhibit inhibitory activities against thromboxane and leukotriene synthesis, both inflammatory mediators (Odontuya et al., 2005). The polyphenols from honeybush have also investigated regarding their anti-inflammatory properties. Mangiferin exhibits inhibitory effects as against the production of TNF-α and IL-6 (Gong et al., 2013). Hesperidin has displayed anti-inflammatory activity in mice by the reduction of pro-inflammatory cytokines release in mice (Tamilselvam et al., 2013).

The activation of role Toll-like Receptors (TLR) conserved transmembrane proteins responsible for detection of pathogens and activation of immune responses, has been described in macrophages following LPS exposure (Bhattacharyya et al., 2007). The stimulation of TLR leads to the production of pro-inflammatory mediators including cytokines and nitric oxide. In most cases, this response was independent of NFκB activity but dependent on one or more of the MAPK pathway components such as ERK, JNK and p38 depending upon both the stimulating agonist and the target TLR. Therefore complex pathways involved in transcriptional regulation of TLR, immune induction and inflammation appear to exist. EGCG has been shown to upregulate Toll-like interacting protein (Tollip), an inhibitor of TLR signalling (Byun et al., 2012). Youn et al. (2006) demonstrated the ability of curcumin, a polyphenol form *Curcuma longa*, to directly inhibit TLRs-mediated signalling pathways.

The role of iron in macrophage-mediated cytotoxicity by contributing to the production of highly toxic hydroxyl radicals via the Fenton reaction and by controlling the production of nitric oxide (NO) after activation by immunologic stimuli is critical (Mulero and Brock, 1999). A moderate increase in the nonheme iron content in hepatic macrophages by erythrophagocytosis, promotes subsequent LPS-stimulated NFκB activation in a hemeoxygenase-dependent manner. Recent evidence also suggests a role of intracellular low molecular weight iron in the early signal transduction for LPS-mediated NF-κB activation (She et al., 2002; Tsukamoto, 2002; Xiong et al., 2003). Similar mechanisms existed when considering exposure of cells

to UVB as it also effect oxidative stress and initiates the activation of transcription factor pathways, which in turn regulate the expression of a number of genes termed the “UV response genes” including the NF-κB and AP-1 families (Cooper and Bowden, 2007). Iron release from ferritin in keratinocytes following UVB exposure has been described and are suggested to play an important role in mediating oxidative stress and subsequent downstream events related to gene expression and cytokine induction (Kramer-Stickland et al., 1999; Pelle et al., 2011). The iron chelating abilities of rooibos and honeybush polyphenols, namely aspalathin, nothofagin and mangiferin, have been described and it is therefore possible that these polyphenols play a major role in the stabilisation of iron, thereby preventing oxidative stress (Andreu et al., 2005; Pardo-Andreu et al., 2006; Snijman et al., 2009).

Modulation of UVB and LPS-induced inflammatory responses

The current investigation into the antioxidant activities of the different tea and herbal tea extracts indicated that radical scavenging properties and the interaction with iron may be important contributing factors in the modulation of inflammatory responses in keratinocytes and macrophages. However, a high antioxidant activity is not always associated with a strong anti-inflammatory property. It would appear that a high antioxidant activity may result in pro-oxidant activity which adversely affects cell viability, resulting in the induction of apoptosis, thereby masking any anti-inflammatory activity. In this regard the extract of unfermented *C. maculata* exhibited the lowest overall antioxidant activity but tend to be superior when considering its anti-inflammatory properties, specifically in the macrophage inflammatory model. This finding could be attributed to the high levels of mangiferin which is known to stabilise iron and thereby prohibiting pro-oxidant activity. This leads us to believe that the stabilisation of iron by polyphenols could play a role in their anti-inflammatory properties whereby the expression of the NF-κB and AP-1 mediators could be modulated. As polyphenols are also known to modulate MAPK signalling it could also be hypothesised that the TLR will be modified via this signalling pathway (Rahman et al., 2006). Several mechanisms of the manner in which herbal teas are likely to modulate inflammatory responses are outlined below (Fig 6.1), aspects that should be further explored in the future.

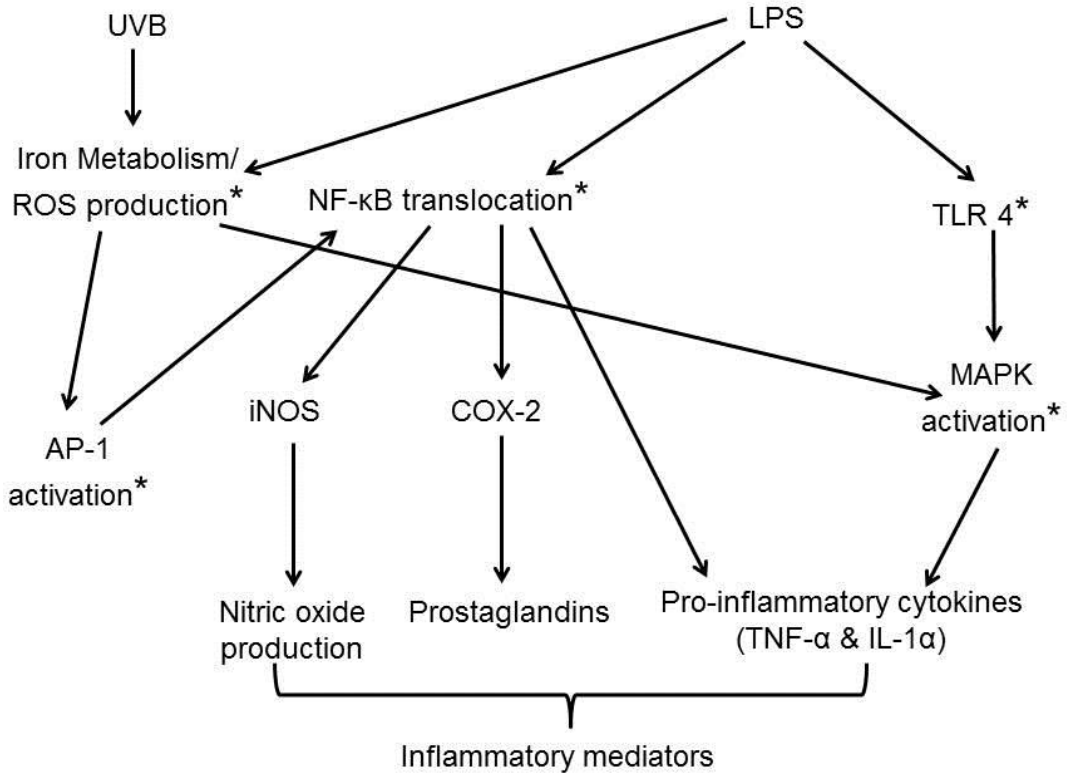

Fig 6.1 Diagrammatic illustration of the pathways involved in the inflammatory process in both UVB exposed keratinocytes and LPS stimulated macrophages. UVB induces the activation of the NF-κB, AP-1 and MAPK pathways in keratinocytes via the release of reactive oxygen species. This leads to an upregulation of the mediators of inflammation. LPS-stimulation of macrophages leads to the activation of the NF-κB pathway, TLR4 and ROS production, which in turn leads to the production of various inflammatory mediators. An asterisk (*) indicates where the herbal tea polyphenols is likely to exhibit their anti-inflammatory properties.

Future studies

In order to further delve into the mechanism of the anti-inflammatory effects of the herbal tea extracts, further studies are required. Western blots can be employed to investigate the modulating effects of the herbal tea on the expression of AP-1, NF-κB and MAPK. Modulation of the mediators of the inflammatory response, such as iNOS

and COX-2 should also be further investigated. Tools to study gene expression, such as real-time polymerase chain reaction can also be utilised to investigate the effect on the expression levels of these inflammatory mediators. Flow cytometry should also be considered for accurate analyses of cell viability, apoptosis and other cell cycle parameters.

Conclusions

As a cancer cell takes a long period to develop and chronic inflammation plays a major role, herbal tea extracts may be useful in controlling skin cancer by delaying the promotion stage and preventing the onset of inflammation. However, the levels of the active polyphenolic compounds in extracts as well as their biological properties should be standardised through a set of *in vitro* antioxidant and cell-based assays which together with the chemical characterisation could be used as quality control parameters to ensure the efficacy of the extracts in skin cancer prevention. Antioxidant, pro-oxidant and anti-inflammatory properties of these extracts should be carefully assessed for the development of possible nutraceuticals for therapeutic application on skin. In this regard specific dose effects of the herbal tea extracts should be investigated in more detail as it is known that U-shape dose-response exists in many biological studies. This became important as low doses of the tea and herbal tea extracts is likely to exhibit anti-inflammatory properties whilst at higher dose adverse effects, associated with pro-oxidant and pro-apoptotic effects, seem to prevail (Fig 6.2).

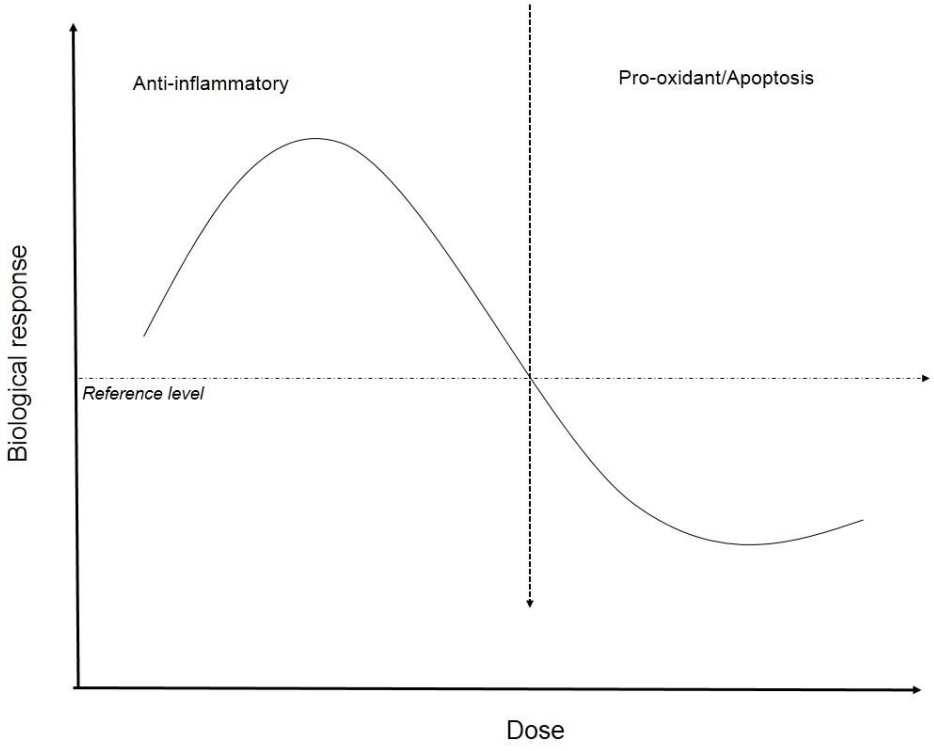

Fig 6.2 Typical U-shape dose-response relationship depicting the effects of the herbal tea extracts against inflammation and the induction of apoptosis.

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